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Peter M. Rabinovich *Editor*

Synthetic Messenger RNA and Cell Metabolism Modulation

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Synthetic Messenger RNA and Cell Metabolism Modulation

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Preface

Synthetic mRNA is an attractive tool for mammalian cell reprogramming that can be used in basic research as well as in clinical applications.

Present mRNA in vitro synthesis is a rather simple procedure, which delivers a high yield of quality product. Various modifications may be introduced into the mRNA by changing the sequence of the DNA template, by modifying the reaction of transcription or by posttranscriptional modification.

mRNA, as a transfection agent, has several advantages over DNA. mRNA expression is not dependent on nuclear entry and occurs directly in the cytosol. More than 90% of the cell population may uniformly express individual or multiple RNAs, just a few hours after transfection. Because of its cytoplasmic location, and in the absence of rare reverse transcription events, mRNA transfer does not affect the integrity of the host genome.

In spite of the obvious advantages of mRNA-mediated reprogramming, some important technical problems still exist and have to be addressed. These are mRNA instability, immune response to mRNA transfection, and inefficient mRNA delivery in some primary cells and tissues.

Topics selected for this volume cover the main methods used, including mRNA synthesis, modifications, and delivery. Examples of cell reprogramming and analysis in the fields of immunotherapy and stem cell research are also included.

This will be of interest to researchers, clinicians, and biotech companies interested in mRNA-mediated cell reprogramming.

New Haven, CT, USA *Peter M. Rabinovich*

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Part I

mRNA Synthesis, Purification, and Transfer

Chapter 1

Cell Engineering with Synthetic Messenger RNA

Peter M. Rabinovich and Sherman M. Weissman

Abstract

mRNA has become an important alternative to DNA as a tool for cell reprogramming. To be expressed, exogenous DNA must be transmitted through the cell cytoplasm and placed into the nucleus. In contrast, exogenous mRNA simply has to be delivered into the cytoplasm. This can result in a highly uniform transfection of the whole population of cells, an advantage that has not been observed with DNA transfer. The use of mRNA, instead of DNA, in medical applications increases protocol safety by abolishing the risk of transgene insertion into host genomes. In this chapter, we review the aspects of mRNA structure and function that are important for its "transgenic" behavior, such as the composition of mRNA molecules and complexes with RNA binding proteins, localization of mRNA in cytoplasmic compartments, translation, and the duration of mRNA expression. In immunotherapy, mRNA is employed in reprogramming of antigen presenting cells (vaccination) and cytolytic lymphocytes. Other applications include generation of induced pluripotent stem (iPS) cells, and genome engineering with modularly assembled nucleases. The most investigated applications of mRNA technology are also reviewed here.

Key words: mRNA, Transfection, Expression, Adoptive immunotherapy, Vaccination, T cells, Chimeric antigen receptor, Cell reprogramming, RNA viruses, Review

1. Introduction

Cell reprogramming by intracellular transfer of macromolecules is a powerful method in cell research and medicine. Although the direct delivery of specific proteins into cells can be quite useful (1, 2), a more universal approach is based on nucleic acid transfer. Unique characteristics of DNA, such as metabolic stability, and the diversity of delivery methods have determined its role as the most widely employed reprogramming agent. Recently, attention has been focused on mRNA as a tool potentially superior to DNA in some applications. To be expressed, exogenous (exo) DNA must be transmitted through the cell cytoplasm and placed into the nucleus. In contrast, exo mRNA simply has to be delivered into the cytoplasm. This often results in a uniform expression of individual or

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multiple exo mRNAs in the whole population of the cells. Such an efficiency has not been observed with DNA transfer (3–5). One potential drawback of mRNA transfer is the instability of mRNA in the cytoplasm. This may be overcome by repeated transfers of mRNA and partly by methods that prolong mRNA expression. The use of mRNA, instead of DNA, in medical applications may increase the protocol safety by abolishing the risk of transgene insertion into host chromosomes. Obviously, this also abolishes the risk of indefinite transgene persistence in the treated cells.

In this chapter, we review aspects of mRNA structure and function that may contribute to the behavior of exo mRNA in cell cytoplasm. This includes the composition of mRNA molecules and their complexes with proteins, mRNA compartmentalization, translation, and the duration of expression. We also review the most investigated applications of the mRNA technology. In immunotherapy these comprise vaccination (by transfection of antigen presenting cells), and also direct reprogramming of cytolytic lymphocytes. Other applications include the generation of induced pluripotent stem (iPS) cells, and genome engineering with modularly assembled nucleases.

This review mainly focuses on materials published during the last 5 years. For further reviews covering these topics see refs. (6–17).

2. mRNA in the Cytoplasm

The majority of mRNA transcripts in the cytoplasm consist of monocistronic molecules that contain a 5′methylguanosine (m7G) cap, a 5′untranslated region (UTR), a coding sequence starting at the first AUG codon, a 3′ UTR, and a polyadenylated (Poly (A)) tail. Production of mRNA in the eukaryotic nucleus is a multistep process that includes synthesis, modification, and often splicing. mRNA leaves the nucleus as a messenger ribonucleoprotein complex (mRNP). The proteins that constitute the mRNP are either attached to the transcript by high affinity RNA binding domains (18) or recruited by small noncoding RNAs (19). These proteins determine mRNP traffic in the cytoplasm, metabolic stability, and the regulation of translation (20, 21). For instance, the "nuclear" cap-binding protein complex (CBC) binds to the 5′cap structure of nascent transcripts and then participates in multiple steps of mRNA processing in both the nucleus and the cytoplasm. In the nucleus, CBC participates in the splicing, 3′end formation and nuclear export. It also mediates the nuclear export of intronless mRNAs (22).

In the cytoplasm, CBC enables a pioneer round of translation. This process monitors mRNA quality and can lead to nonsense-mediated mRNA decay (23). It may also produce the peptides for "self antigen presentation" via the MHC class I pathway (24) (also see Subheading 6.1.3). During and after the pioneer round of translation, "protein remodeling" of mRNP occurs. As a result, a number of "nuclear" RNA binding proteins are either removed or replaced with their cytoplasmic counterparts. For instance, nuclear CBC is replaced with the cap-binding factor eIF4E, while the nuclear Poly(A) binding protein (PABP) N1 is replaced with PABP C1 (23).

The remodeled mRNAs exist in the cytoplasm as free mRNP particles, as parts of the translational machinery, or in various RNA-containing granules that are involved in mRNA transport, storage or decay (25).

2.1. Translation Efficiency

Given the complexity of translation, multiple factors affect its efficiency. These include mRNA localization, codon usage, size of tRNA and nucleotide triphosphate pools, availability of ribosomes and translation factors, etc. Among these, the codon usage is a parameter that can be controlled in a synthetic mRNA preparation.

It is assumed that the use of abundant codons increases the efficiency and fidelity of translation. At the same time, the introduction of rare codons in the 5′proximal part of mRNA can also be beneficial. Analysis of multiple genomes has shown a trend in which the first 30–50 codons in genes correspond to more rare tRNAs (26). Such codon distribution may slow down the ribosomes in early elongation, prevent nascent protein misfolding, attenuate ribosomal "jamming" and fall-off (27).

In rapidly induced genes, the neighboring codons of the same amino acid are often translated by the same cognate tRNA. It has been speculated that the acceleration of translation of such genes can be caused by the presence of tRNA recharging machinery in the vicinity of translating ribosomes (26).

Translation can also be controlled by *cis*-regulatory elements located in transcripts that are capable of attracting trans-regulators. Some of these trans-regulators are proteins, while others consist of both proteins and noncoding RNAs, particularly miRNAs. (16). Most trans-regulators are inhibitory molecules that suppress translation by various mechanisms, such as blocking initiation of translation or preventing mRNA pseudo-circularization (11, 13, 28, 29), although in some cases they may increase translation efficiency (30, 31). Regulators often bind to the 3′UTRs, and less often to the 5′UTRs or the coding regions (11, 13, 32). During translation, the 3′UTR is the only part of mRNA which is not directly involved in ribosome binding and, in turn, more available for trans-acting regulators. Thus, one may argue that the regulatory *cis*-elements, active during translation, should preferably be located in 3′UTRs. Conversely, *cis*-elements, active in non-translated mRNAs, should be more evenly distributed among the mRNA domains.

2.1.1. Initiation of Translation

To be competent for translation initiation, mRNA must contain the appropriate flanking elements. In eukaryotic mRNA, both ends interact with specific proteins, as follows. The 5′cap binds to eIF4E, then to eIF4G and eIF4A factors, forming the eIF4GF complex, while the 3′-terminal Poly (A) tail adheres to the Poly(A)-binding protein (PABP) (13, 33). Subsequently, the 5′eIF4F and 3′PABP bind each other to form a "pseudo-circularized" structure. This molecule further recruits the 43S ribosomal preinitiation complex, composed of a 40S small ribosomal subunit, GTP, Met-tRNA, and translation initiation factors (11, 34). Circularization provides quality control of mRNA by monitoring the presence of the functional 5′cap and 3′Poly(A). Additionally, because it brings together sites where ribosomes enter and leave mRNP, the circularization may increase translation rates by facilitating the reloading of ribosomes (35).

1. *Translation initiation with 5′ UTR scanning*. The 43S preinitiation complex scans the mRNA 5′UTR until it recognizes an AUG start codon via base-pairing with Met-tRNA. Next, it binds to the 60S large ribosomal subunit to form the 80S initiation complex that enters the peptide elongation phase (11, 15).

   In general, 43S scanning terminates at the 5′ proximal AUG codon, although sometimes it may continue to a downstream AUG (leaky scanning). To prevent leaky scanning, the first codon should be presented in the context of the Kozak element: A/G CC**AUG**G, and be placed at some distance from the 5′end. A minimum of 20 nt 5′UTR is required for efficient recognition of the first codon, whereas a shorter 5′ UTR gives rise to leaky scanning (15). Usually, the 5′UTR lengths range between 50 and 200 nt (15, 36).

   The 5′UTR may contain *cis*-elements that regulate translation, such as the iron response elements (37), 5′terminal oligopyrimidine tracts (38), and miRNA binding sites (16). A strong secondary stem-loop structure may inhibit translation by blocking the binding of 43S to the 5′cap or by preventing 5′UTR scanning (39). However in some cases, the secondary structure can be a target sequence for proteins that enhance translation (30).

2. *Translation initiation without 5′ UTR scanning*. Short 5′UTRs with a median length of 12 nt preceding the AUG codon have been found in a portion of mammalian mRNAs. Translation initiation at short 5′ UTR (TISU) elements enables efficient 5′cap-dependent translation without scanning (15, 40). This is independent of eIF4A, an RNA helicase that facilitates scanning. A moderate stem-loop structure downstream of the starting AUG codon enhances translation fidelity, probably by facilitation of 43 s binding in the absence of sufficient 5′ UTR contacts (40).

3. *Internal ribosome binding sites (IRESs). IRESs* mediate 5′end-independent initiation of translation in some cellular and viral mRNAs (41, 42). The level of translation initiation at these sites is usually lower compared to conventional initiation. Although 5′capping is not required for IRES function, translation of mRNAs that contain IRESs at the 5′ends can be enhanced by capping (43). This implies that these mRNAs may also be translated by the cap-dependent 5′UTR scanning mechanism. During normal growth conditions 5′cap dependent translation of such genes is preferred. Stress attenuates translation via phosphorylation of eIF2a, and this switches IRES-mediated translation to a 5′cap independent mode (44).

2.1.2. Coding Region

Cis regulatory elements in coding regions can enhance or repress translation (32). A weak stem-loop structure downstream of the starting AUG codon may enhance translation fidelity via ribosome pausing that facilitates the formation of the elongation complex (15). The presence of splicing junctions in transcripts can enhance translation. These transcripts leave the nucleus bound to the exon junction complex (EJC) proteins (45, 46), and are capable of a higher rate of translation than non-spliced controls. How EJC protein can enhance translation is unknown. They may facilitate the recruitment of ribosomes for steady-state translation or mediate the trafficking mRNA toward translationally active locations. A similar stimulation of translation can be obtained for non-spliced mRNA by tethering of three EJC proteins (47).

2.1.3. 3′ UTRs

The 3′UTR is the most important *cis* element in determining mRNA stability, localization, and efficiency of translation (48). The 3′ UTRs of many eukaryotic mRNAs form tertiary nucleoprotein structures with the coding regions, presumably through interaction with translating ribosomes and regulatory 3′UTR binding proteins (49). The average length of 3′UTRs has increased during evolution (50, 51) and reaches about 800 nt in vertebrates (52). The 3′UTR *cis*-regulatory motifs may contain various sequences, such as U-, AU-, GU-rich elements (30, 32) and the majority of sites binding miRNAs (53).

In addition to their *cis*-regulatory function in mRNA, some 3′UTR sequences may act as independent regulatory molecules. Transcripts arising from the 3′UTRs but lacking the upstream coding sequences have been detected in a number of cell types. Such “free” 3′UTR sequences may originate as products of mRNA degradation, or they may be independently expressed (54, 55).

2.1.4. Poly(A) Tail

An average mRNA contains ~250 adenylic acid residues on its 3′end, which is covered by Poly(A) binding protein (PABP). This protein binds to the Poly(A) tail in a cooperative manner yielding a repeating pattern of binding of ~27 nt (35), or about 10 molecules of PABP per 1 molecule of mRNA (35, 56). PABP stimulates translation of capped mRNA by connecting the Poly(A) tail to the 5′cap. This interaction can be emulated with exo mRNA that lacks a Poly(A) tail by tethering PABP to the 3′UTR (57). A somewhat similar situation occurs with replicative histone mRNAs, that naturally lack 3′Poly(A) tails. These mRNAs have a stem-loop structure at their 3′UTRs that, via the stem–loop binding protein (SLBP), interacts with 5′caps, leading to the "pseudo-circularization" of the mRNAs (58).

2.2. mRNA Compartmentalization

Localization of mRNA in certain cytoplasmic regions or organelles can have important functions, such as promoting site-specific control of mRNA activity or accumulating proteins at specific sites (59). mRNA distribution through the cytoplasm occurs by passive diffusion or active transport (59). The specificity of mRNA sorting is often determined by Zip regulatory elements that are present in the transcripts (60, 61). These elements are typically located in the 3′UTRs, but may also reside in the 5′UTRs or in the coding regions (62, 63), including exon junctions (64). Zip elements participate in mRNP assembly with adaptor proteins. Adaptors attach mRNPs to molecular motors that transport them along the cytoskeleton to their destination (20, 65). Some mRNAs can be transported as single RNPs, while others are assembled into transport particles containing multiple mRNAs (66, 67). A large number of transcripts, such as β-actin mRNA, can be transported through the cytoplasm in translationally silent form to be activated at their destination points (65, 66).

Among various cellular compartments, two regions are relatively easy to distinguish biochemically: the cytosol and the endoplasmic reticulum (ER). ER is the predominant site for translation of ~2/3 of cytoplasmic mRNA. It contains almost all mRNAs encoding transmembrane proteins, most of the secretory proteins, and many cytosolic proteins (68). Secretory proteins often contain amino-terminal signal peptides that target proteins to the ER during translation. The signal peptide recognition particle is formed on nascent polypeptide chains, anchors the ribosome on the ER and transports newly synthesized protein across the membrane (69). Some of the ER resident mRNAs show translation-independent association to the ER. They include mRNAs encoding cytosolic proteins that do not contain signal sequences. Also, some mRNAs encoding signal peptides would not leave the ER even if signal peptide sequences are deleted or mRNA translation is blocked (70). The exact mechanism that determines the location of these mRNAs is unknown.

Non-translated mRNA can accumulate in the cytoplasmic non-membranous granules, such as P-bodies, which generally contain the mRNA decay machinery, and the stress granules (SGs) which contain some translation initiation proteins (71). Another structure, the ALK granule (AGs), has been found in transformed cells expressing the ALK tyrosine kinase. AGs contain polyadenylated mRNAs, PABP and other factors, but not the mRNA degradation proteins (72). It has been hypothesized that mRNA dissociated from ribosomes can be stored in SGs, AGs or similar structures, and under appropriate conditions mRNA can be moved back to the translation machinery (25, 72).

2.3. mRNA Stability

Global quantification of mammalian gene expression has shown that an average cellular half life for mRNA is 9 h, and 46 h for proteins. Proteins are, on average, five times more stable and 900 times more abundant than mRNAs (73). Short-lived mRNAs usually encode highly reactive proteins, such as cell cycle control factors and cytokines. mRNAs encoding abundant proteins such as globins, collagens, crystallins and immunoglobulins, are more stable (74). The level of individual mRNAs in the cytoplasm is maintained by the balance between mRNA entry and decay. mRNA can be degraded by 5′-3′ exonucleases, by 3′-5′exonucleases, or by endonucleolytic attack. The decay of most mRNAs is exonucleolytic, and therefore, the end structures of mRNA are important determinants of stability (75, 76).

Translation is usually considered as a factor that increases mRNA stability. On the other hand, translation itself can be coupled with degradation of transcripts. Usually the decay of polysomal mRNA is initiated by the shortening of the poly(A) tail. This is controlled by the cooperative activity of a multiprotein complex that includes PABP and deadenylases (77, 78).

When the Poly(A) tail degrades to ~20–30 nt, e.g., when mRNA cannot effectively bind PABP molecules, initiation of translation is hindered and mRNA may enter the degradation pathway. In this way, mRNP loses proteins that are involved in translation, such as eIF4G, and recruits translational repressors and decapping enhancer proteins (33, 75). Removal of the cap is considered to be the “decisive” step in the mRNA degradation pathway, yielding 5′-monophosphorylated mRNA. Further reactions include 3′-5′ and 5′-3′-exonucleolytic and, sometimes, endonucleolytic degradation. This usually takes place in the P-body compartment, although mutations that prevent P-body formation do not lead to increased mRNA stability (75, 79).

Elements that modulate stability of mRNA most often reside in the 3′UTRs, and less often in the 5′UTRs or in the coding regions. In the 3′UTRs, decay factors target U-, AU-, and GU-rich, and similar elements (32, 80), 3′UTR iron responsive elements (37), and many sites involved in the miRNA guided silencing (28, 53, 81, 82).

In the 5′UTRs, the target sequences include polypyrimidine tracts (83) or miRNA binding elements (32, 53, 84). The target sequences in the coding regions can modulate mRNA stability by direct binding of specific proteins or by interaction with miRNAs (32). Long noncoding RNAs also can target mRNA in the cytoplasm directly, or as siRNAs, after Dicer-mediated degradation (81, 85).

RNA fragments that arise from mRNA cleavages can create duplex structures that are processed into small RNAs by Dicer endonuclease. Subsequently, these products may act as regulatory elements of translation (82, 86).

3. Expression of exo mRNA

The expression of exo mRNA is determined by the efficiency of delivery, the rate of translation and stability of the mRNA. After the method of mRNA transfer (Subheading 4) is chosen, the next step would be the optimization of mRNA structure that fits both the type of targeted cells and the method of delivery. All structural elements of natural mRNA, such as the (m7G) cap, 5′- and 3′ UTRs, and Poly(A) tail are important for expression of exo mRNA. However, the details of exo mRNA processing in the cytoplasm remain to be elucidated. It would be important to find the composition of RNA binding proteins and the sequence of their attachment to exo mRNAs, the trafficking of exo mRNPs in the cytoplasm, and their distribution among ribosomal pools, as well as how particular methods of delivery influence mRNA distribution in cytoplasmic compartments.

The rate of translation and mRNA stability are critical factors affecting the duration of mRNA expression. To date, much attention has been focused on increasing the resistance of mRNA to degradation. One approach is to chemically protect the ends of mRNA from degradation. Chemical modification of the 5′cap can significantly increase mRNA resistance to decapping enzymes and increase the efficiency of mRNA translation (87). Modified nucleotides can be attached to the 3′end of the Poly(A) tail to prevent its degradation (5).

Another approach is to use 3′UTRs obtained from “stable” mRNAs. For example, the introduction of the repeat of β-globin 3′UTR or the multiple repeats of 3′UTR from Venezuelan equine encephalitis virus mRNA increased exo mRNA stability (88, 89). It should be noted, however, that the flexibility of degradative mechanisms may overcome end-protective modifications of the mRNA, for instance, by triggering an endonucleolytic cleavage (90).

An alternative and yet speculative idea to prolong mRNA expression is to adopt the cytoplasmic re-capping and polyadenylating enzymes for the restoration of mRNA ends. Both enzymatic activities have been found in the cytoplasm. The cytoplasmic re-capping complex contains a kinase that, together with a capping enzyme, converts the 5′-monophosphate RNA into 5′-GpppX RNA (33, 91). Cytoplasmic polyadenylation in eukaryotes regulates the length of mRNA Poly(A) tails during the cell cycle. This mechanism involves the recruitment of cytoplasmic poly(A) polymerase to the *cis*-acting polyadenylation elements in the 3′ UTR (92, 93). It would be advantageous to use the cytoplasmic enzymes to reverse the exo mRNA degradation.

Another alternative approach is to use RNA viral vectors that are able to persist in the cytoplasm and provide long-term expression of the mRNA of interest, by continuous cytoplasmic transcription (see Subheading 4.5).

4. mRNA Delivery

The goal of mRNA delivery is to provide the safe and efficient transfer of mRNA to target cells. In vivo this can be achieved by local or systemic delivery. In addition, some types of cells, such as lymphocytes and dendritic cells, can be isolated from the host, propagated ex vivo, reprogrammed with mRNA in vitro, and transfused back to the host. Both in vivo and in vitro methods of mRNA transfer are based on the same mechanisms of mRNA uptake and usually adapted from methods developed for delivery of DNA. Often, the DNA transfection protocol can be applied to mRNA without any adjustment. For example, comparisons made on different cell types concluded that the optimal regimes of electroporation are identical for mRNA and DNA (4, 5). However, other methods that use nanoparticles, liposomes, and viral particles need to be specifically optimized for mRNA delivery.

Whatever methods of mRNA transfer are used, their invasiveness cause a certain level of stress, and leads to the perturbation of global transcription in the cells. In addition to changing the transcription rate of multiple genes, cell stress also changes the decay rate (94) and translation efficiency (95, 96) of stress-responsive mRNAs. Different methods of mRNA delivery may have different consequences depending on the specific stress reactions they cause.

The evaluation of the effects of cell stress reactivity on mRNA transfer is quite important, because the duration of mRNA expression is relatively short and a substantial part of it may overlap with the process of cell recuperation from the transfer procedure. In some cases, such as in reprogramming of dendritic cells with antigen-coding mRNAs, a certain level of cell stress can be beneficial because it may facilitate antigen processing and cross-presentation.

4.1. Naked mRNA

The most straightforward approach for mRNA delivery in vivo is the injection of naked mRNA into the tissue. Despite concerns regarding mRNA stability, it has been demonstrated that mRNA can be efficiently introduced and expressed in skin, muscle, and lymph nodes (97–99). Apparently, the main mechanism of mRNA uptake is endocytosis (100). Naked mRNA transfer might be difficult to achieve in cultured cells because of electrostatic repulsion between the negatively charged cell membrane and mRNA. It is speculated that the injected mRNA attracts some charge-neutralizing factors, such as calcium (98) and probably cationic peptides (101, 102), and that this facilitates mRNA attachment to the cell membrane. In vitro, mRNA uptake was demonstrated with dendritic cells, which can uptake polycationic molecules by macropinocytosis (99).

4.2. Electroporation

This method has been developed for intracellular transfer of macromolecules, such as DNA, RNA and proteins. mRNA electroporation has been evolved as an adaptation of the technique used for DNA electroporation. When an electrical field surrounding the cell increases to the point that exceeds the membrane potential, this leads to depolarization of the membrane at both poles of the cell that face electrodes. Small molecules in the vicinity of such a cell, may penetrate into the cytoplasm at both poles through multiple small pores in the cell membrane (103, 104). Nearby negatively charged macromolecules, such as DNA and mRNA can be electrophoretically moved toward the cells, bind to the cell surface and then penetrate it at the pole facing the cathode. Apparently, DNA enters the cytoplasm not through membrane pores which are too small for them, but rather by unknown mechanism (104). The recuperation of the cellular membrane after DNA electroporation can require as long as 50 min (105). The cellular stress caused by electroporation may continue even longer.

Compared to DNA, mRNA electroporation is more efficient, as more than 90% of the cells in the population can uniformly express single or multiple exo mRNAs (5).

The regime of electroporation depends on multiple factors, such as cell diameter, cell membrane content, ionic strength of the medium, and the presence of surfactants and other substances that may alter membrane permeability. Unfortunately, the most recently developed commercial systems do not disclose the parameters of electroporation. Nevertheless, it is safe to assume that, in most cases, one or a few square wave impulses of the same polarity will produce satisfactory results. Cell toxicity, besides cell membrane perturbation, is often caused by toxic ions extracted from the aluminum electrodes used in most electroporators. The use of electrodes made from noble metals, and physical separation of the cells from the electrode surfaces increase viability of the cells (106, 107). Large volume electroporators with nontoxic electrodes are able to electroporate cells in continuous flow units. These systems can transfect hundreds of millions of cells in minutes with high efficiency and viability (108, 109).

4.3. Nucleofection

This process was introduced by Amaxa (Lonza). The system electroporates the cells suspended in special (proprietary) solutions and is reported to transmit DNA not only through the cell membrane, but also through the nuclear envelope (110). This method employs a combination of proprietary programs that are optimized for different cell types. It has been very effective in the delivery of DNA, mRNA and small RNAs to various types of cells, including many primary cells (111). Side-by-side comparison of the conventional electroporation and nucleofection for nucleic acid transfection showed that both methods work well and give similar results for "easily transfected cells," i.e., cells that can be efficiently transfected by conventional electroporation. However, cells that are difficult to transfect by conventional electroporation can often be successfully transfected by nucleofection (5, 112).

4.4. Nonviral Particles

This includes a very diverse group of carriers, different in chemical structure, shape, mechanisms of mRNA binding and release, stability, and toxicity (6, 7, 113). The particles are usually several hundred nanometers in scale. This size is important for their colloidal stability in physiological solutions, and their interaction with the cell membrane. The carriers protect mRNA from degradation and mediate its transport into the cytoplasm. They enter cells mainly by endocytosis, followed by endolysosomal escape and unpacking of mRNA. The most common carrier materials are cationic lipids and polymers, which attract and condense negatively charged mRNA, forming lipoplex or polyplex complexes that are able to exist as stable suspensions. The performance of lipoplex and polyplex particles depends on multiple parameters, such as the chemical type, topology of the cationic polymer, integral charge, and the affinity to cell surfaces. To escape from the endosomal compartment, particles may contain pH-sensitive moieties. These moieties, when activated by endosomal acidification, trigger osmotic swelling and membrane disruption which release the cargo into the cytoplasm (113, 114). The carriers may include linear and branched polymers, and their combinations, cell targeting ligands, and additional moieties that define hydrophobicity, charge distribution, size, and stability of the particles. Much effort has been focused on developing carrier systems that are suitable for clinical use (115–118). A new delivery system that employs yeast cells as the vehicle has been recently proposed for dendritic cells (119).

4.5. Viral Particles

Viruses with RNA based genomes that replicate exclusively in the cytoplasm have been explored as potential vectors for introducing mRNA into cells. Among the important requirements for such vectors are their safety, and lack of cytotoxicity. To achieve this, some essential viral genes must be deleted. Consequently, such vectors are propagated in packaging cells, containing the complementary viral genes that are absent from the vectors (8).

Propagation-proficient vector systems allow viral multiplication. These may be applied to construct cytotoxic vectors for anticancer therapy, although such an approach is usually associated with high safety concerns. More often, for safety reasons, special efforts are made to maintain the vectors' inability to propagate and to spread among non-targeted cells.

Two of the most developed vector systems are based on positive single stranded and negative single stranded RNA viruses. Positive single stranded RNA viruses contain RNA which can be directly translated by host translation machine. Negative single stranded RNA viruses contain "antisense" RNA packed in nucleoprotein–RNA complex, which, prior to translation by cell ribosomes, must be transcribed by viral transcriptase. Both viral systems can target a wide variety of mammalian cells, but are markedly different in regards to the methods of vector engineering and the fields of application.

Positive single stranded alphavirus vectors can be constructed by conventional genetic engineering methods that include the production of recombinant viral particles in cells transfected with naked vector RNA. The recombinant alphaviruses enter target cells by endocytosys (120, 121). Some of these vectors show significant neurotropism and tumor targeting capabilities. This makes them attractive tools for the treatment of CNS-related diseases and cancer (122).

In the case of negative single stranded RNA vectors, the engineering technique is more complicated. Naked vector RNA cannot be rescued without specific viral replicase, and the efficiency of rescue for each new construct is relatively low. However, recombinant viruses have some advantages. For instance, they can provide stable persistence and expression of the exo RNA. Sendai virus (SeV) adsorbs to neuraminic acid containing cell surface components, and can penetrate cells without endocytosis (123). SeV is responsible for a respiratory tract infection in mice and some other animals, but causes no disease in humans. Recombinant SeV vectors show low levels of cytotoxicity (124), and can therefore be used for various therapeutic applications, including cancer treatment (125).

Recently, Nakanishi and colleagues have reported generation of a defective SeV vector that stably expresses up to four exo genes in infected cells (also see Subheading 6.3.1). The viral vector lacks genes for capsid formation and therefore cannot be rapidly transmitted from cell to cell. Mutations in the nonstructural proteins of the virus enable long term persistence of the vector, possibly, in part, by preventing interferon induction. The viral RNA-nucleoprotein complex can be replicated within the cytoplasm of infected cells, and this contributes to a very lengthy persistence of this vector (126).

Another delivery system made out of SeV contains no viral genetic material at all. "GenomeONE virosomes" (CosmoBio), contain only structural viral proteins. According to the vendor, these particles can deliver siRNA and proteins into various types of cells (127), and can potentially be used for mRNA delivery. However the conditions for mRNA transfer have not yet been established.

Retroviral vectors also can be adopted for mRNA delivery. Retroviruses are positive stranded RNA viruses that replicate in the nucleus via DNA replicative intermediates. If reverse transcription of viral mRNA is abolished by mutagenesis, the virus loses the ability to replicate, and viral particles can be used for cytoplasmic delivery of exo mRNA. Such vectors can be conveniently produced in well-developed retroviral packaging systems, and modified for targeting of specific cell types (8, 128).

5. Innate Immune Reactions to Exo mRNA

Most cell lines and many primary cells such as lymphocytes, usually readily tolerate the transfection with mRNAs. However, mRNA transfer in other cells, especially in fibroblasts and kerationocytes, can induce a strong immune response. Exo mRNA has routinely been produced in vitro as a single strand polymer containing four unmodified ribonucleotides. The efficiency of capping and polyadenylation can vary, so the product may contain some uncapped molecules or molecules without Poly(A) or with short Poly(A) tails. Also, runoff activity of RNA polymerase may produce double stranded RNA contaminants. Non-capped, insufficiently Poly(A)-tailed mRNAs, and double stranded mRNAs comprise patterns which can be recognized by mammalian cells as foreign (viral or microbial) and induce innate immune response (129–132). The pattern recognition receptors include two types of sensors, namely, the Toll-like receptors—TLR3, TLR7, and TLR8—that are located in the ER, endosomal, and lysosomal membranes, and the RIG-1-like receptors—Rig-1, MDA5, LGP2—that are located in the cytoplasm. If exo mRNA enters the cytoplasm by endocytosis (naked mRNA, nonviral particles, and most viral vectors), it may initially activate endosomal and then cytoplasmic sensors. If mRNA enters the cytoplasm directly without endocytosis (electroporation, SeVvectors) it may stimulate only cytoplasmic sensors (133–135).

The cell immune response to exo mRNA, mediated by inflammatory cytokines and interferons, may sensitize the cells to subsequent exo RNA exposure (130, 136). Foreign mRNA can also activate another cytoplasmic sensor, interferon-inducible RNA-dependent protein kinase (PKR), which results in the global suppression of protein synthesis (137, 138).

The immunogenicity of in vitro made mRNA can be significantly decreased by the introduction of specific nucleotide modifications, and purification from non-modified contaminants (129, 130, 139). Substitution of 5-methylcytidine for cytidine, pseudouridine or

2-thiouridine for uridine, and dephosphorylation, and exonuclease degradation of non-capped mRNA, can alleviate RIG-1 and TLR-mediated interferon signaling and PKR mediated repression of translation. Consequently, this will increase the viability of the transfected cells (129, 130, 140–142). The use of immunosupressants, such as the interferon inhibitor B18R can yield an additive effect (130).

Another way to eliminate immunogenicity of mRNA transfection is the siRNA-mediated repression of the genes involved in immune responses. Combined knockdown of InterferonB, PRK, and Stat2 genes enables repeated transfection with non-modified mRNAs (136).

6. Applications

To date almost all practical applications of mRNA transfer, except viral transduction, benefit from two fundamental advantages: the ability to transfect the cells with a minimal (vector-free) expression cassette, and the ability to transfect virtually the entire population of target cells.

6.1. Vaccination

6.1.1. Cell Reprogramming

mRNA-directed humoral and cellular immune responses have been shown in preclinical model experiments. In clinical trials, vaccinations against cancer and allergy were shown to be possible, effective, and safe (143–145). mRNA can be used alone or complexed with protamine, and can be used for intradermal, subcutaneous, intranodal, and intravenous applications (144, 146). Another approach is to use mRNA for ex vivo reprogramming of antigen presenting cells, primarily dendritic cells (14, 144, 147, 148). B cells can also be employed (149).

Non-modified mRNA stimulates strong immune reactions through RIG-1-like and Toll-like receptors (see Subheading 5). The additional transfection with mRNA coding for IL12, CD40-L, TLR4, CD70, OX40-L, or GM-CSF substantially improves the outcome (14, 147). Co-transfection with siRNAs targeting anti-inflammatory pathways can also facilitate mRNA-mediated vaccination (14).

6.1.2. Monitoring of Immune Responses

mRNA transfection can be used not only for reprogramming of antigen-presenting cells, but also for efficient monitoring of antigen-specific immune responses and analysis of MHC class I and II epitopes (150, 151).

6.1.3. Antigen Presentation

It has been suggested that MHC I antigen presentation mainly depends on the degradation of full length proteins, the products of steady state translation. However, recent work suggests that the main pathway for MHC I antigen presentation depends on the products of the pioneer round of translation—which produces the peptides using "non-matured" mRNPs that have been recently exported from the nucleus and contain large amounts of aberrant RNA (152). It is generally accepted that this mechanism has developed as a defense against harmful (e.g., viral) invaders and that it allows cells to present antigen determinants before large amounts of the full cognate proteins are translated and become active (24, 152).

Also it was shown that transfected non-spliced mRNAs determine the synthesis of antigen peptides only early after transfection. Probably this suggests that the protein synthesis responsible for antigen presentation precedes steady state translation not only for spliced, but also for not-spliced mRNAs (152).

The presentation of the MHC class II epitopes depends on their availability in the endosomal compartment. The product of the exo mRNA can enter the MHC II pathway by different mechanisms. For example the protein can be secreted and then enter antigen presenting cells by endocytosis. In cross-presentation the antigens derived from exo mRNA translation are redirected to the class II pathway (153, 154).

6.2. Cytolytic Lymphocytes

T and NK cells, are attractive targets for combining cell engineering with immune therapy for the treatment of various pathologic conditions such as cancer, allergy, and acute infections. Cytolytic lymphocytes have endogenous killing potential and diverse specificity determined by their immune receptors. The main approach in cell reprogramming is to uniformly express a new receptor that redirects the entire lymphocyte population toward a specific target. In addition, the treatment should preserve or improve the cells' essential effector functions, such as cytotoxicity, viability, and motility.

The list of exo mRNAs that have been used includes:

Anticancer chimeric antigen receptors (CARs), such as CD19 CARs (5), B7-H3 (8 H9) (155), Her-2/neu CAR (156);

Anticancer TCR receptors against NY-ESO-1, MART-1, and p53 (157);

Chemokine CXCR2 receptor(158);

IL-15RA receptor and its fusion with IL15 (159).

CAR is a monomolecular construct that contains a recognition domain, usually a single chain antibody, a transmembrane domain, and signaling domains, such as the intracellular domain of the ζ-subunit of T cell receptor (TCR), or the signal domain of CD137 protein, among others (160–163). Another design for lymphocyte reprogramming employs the transfer of two molecules: α and β subunits of the target-specific TCR (161). It is often easier to obtain TCR constructs of a certain specificity by using natural T cells selected against specific targets. However, CAR proteins can possess cytotoxicity toward a variety of targets in a non-MHC-restricted manner and therefore can be applied to patients of all MHC haplotypes (162). Consequently, the treatment with lymphocytes expressing CARs would not be compromised by the loss of MHC alleles by target cells. Additionally, in contrast to TCRs, CARs can recognize not only target peptides but any antibody-recognizable antigens present on cells, for instance, carbohydrate or glycolipid moieties.

As in many other cases, mRNA reprogramming of lymphocytes uses protocols similar to those developed earlier on DNA models. The methods of T cell isolation are identical for DNA and mRNA mediated reprogramming. T cells can be isolated from peripheral or cord blood and are usually non-specifically activated by combinations of CD3, CD28 antibodies and interleukin. They can be propagated ex vivo for weeks without losing their ability to kill target cells. The culture conditions used during cell propagation affect the state of cell differentiation. T cells are often cultured in the presence of IL-2. This can result in development of a late effector state with low propagative ability. Replacement of IL2 with IL-7 and/or IL-15 produces less differentiated T cells that are capable of long persistence in vivo (164, 165).

Almost an entire population of isolated lymphocytes can acquire the uniform expression of a CAR protein after exo mRNA electroporation. To maximize the advantages of the method, the RNA transfer is usually applied to autologous cells. mRNA transfer can be applied to the whole population of T cells with the potentially synergistic benefit of their physiological diversity. The activation of T cells for mRNA transfection is crucial (155, 166), probably due to morphological or metabolic changes, including translation enhancement or membrane remodeling. However, the actual role of these various factors requires further investigation. Treatment with CAR mRNA-transfected CTLs (CD3+ CD8+ T cells) inhibits tumor growth in vitro and in vivo (6, 155, 167–169). It is worth noting that an approach that does not cure the disease in an immune-deficient host may potentially lead to a better outcome in an immune-competent environment.

Although the main targets for mRNA transfer are CTLs, other lymphocyte subsets tested also showed good response and specific cytotoxicity after reprogramming CD3+ CD4+ T cells (155, 156, 158, 170), cytokine-induced killer (CIK) cells (171), and NK cells (108, 155, 172, 173).

Cytokine-induced killer (CIK) cells are usually produced by ex vivo expansion of cytotoxic T cells in the presence of the anti-CD3 antibody, IL 2, and interferon (IFN)-γ. These cells express NK receptor NKG2D on the surface, and show functional and phenotypical properties similar to those of the natural killer (NK) cell. CIK cells reprogrammed with CARs show high level of cytotoxicity against specific targets (171, 174).

6.3. mRNA Mediated Modulation of Nuclear Events

Although exo mRNA is unable to penetrate the nuclear envelope, it may code proteins capable of genome targeting. These proteins can perform various functions, such as epigenetic modifications of chromatin or targeted cleavage of chromosomes to induce site-specific recombination.

6.3.1. Induced Pluripotent Stem Cells and mRNA Reprogramming

The reprogramming of differentiated cells to pluripotency was first achieved by Yamanaka and colleagues. They used retroviral vectors that expressed four transcription factors KLF4, c-MYC, OCT4, and SOX2 (175, 176). Because of the transient nature of such reprogramming, the idea of replacing viral vectors with exo mRNAs has been considered feasible (130, 177, 178). Most efforts were focused on repeated mRNA transfection of primary cells, which also allows the continuous reprogramming through the whole process of cell de-differentiation. Apparently, human fibroblasts and keratinocytes, which have been used in these experiments, are highly sensitive to the immunogenicity of the synthetic mRNA. Warren and colleagues found that mRNA with low immunogenicity can be produced in vitro as capped molecules containing modified pyrimidine ribonucleotides (130) (also see Subheading 5). This approach allowed multiple transfections of human fibroblasts and keratinocytes with a "cocktail" of mRNAs coding Yamanaka factors (*Oct4/Sox2/Klf4/c-Myc*). This method is simple, safe and because of the efficiency of the multiple mRNA transfection, results in up to 4.4% iPS cell conversion, the highest level of efficiency that has been achieved (130, 179).

Another approach is based on the use of a novel replication-defective SeV. The vector RNA can accommodate *Oct4, Sox2*, *Klf4*, and *c-Myc* factors. Because of the transcription polarity in Sendai genomes, the expression of each gene is position-dependent, with predominant expression of more 3′ proximal sequences. The expression stoichiometry of Yamanaka factors was optimized by adjusting the position of their coding sequences. The transduction of mouse primary fibroblasts resulted in ~1% yield of iPS cells. The propagation of the SeV in iPS cells was terminated after the introduction of a siRNA that interfered with viral transcription (126).

6.3.2. Genome Targeting

Zinc-finger nucleases (ZFNs) and Transcription Activator-Like (TAL) Effector Nucleases (TALENs) can introduce double-strand breaks in specific DNA sequences with high frequency and induce targeted genome mutagenesis or targeted gene replacement. Both ZENs and TALENs contain the sets of independent modules which can be designed to recognize a single target sequence, even in a complex mammalian genome (180–182). This approach is technically complicated and dependent on the empirical adjustment of the constructs to particular sequences. Additionally, the structure of chromatin can prevent cleavage of some loci (180, 183).

However, the broad range of specificities of the modularly assembled enzymes makes them a universal tool for the directed mutagenesis. The use of mRNA as a coding substance increases the efficiency of this approach (184–188).

7. Future Directions

Because of its efficiency and safety mRNA transfer is becoming increasingly appreciated in cell engineering. Short-term expression of mRNA, often an undesirable feature, brings an obvious advantage, if the short-term treatment is sufficient or if multiple treatments are possible. mRNA transfer can be useful in adoptive immunotherapy of cancer, acute infections, and allergies. It is also useful as a tool for modulating cell differentiation and correcting genetic disorders.

Controlled translation and long cytoplasmic persistence of exo mRNAs are key future goals. The ability of a new generation of SeV vectors to exist in the cytoplasm with low or no cytotoxicity opens a new avenue in developing long-lasting mRNA persistence (see Subheadings 4.5 and 6.3.1). A more traditional non-viral approach may include the use of multiple modifications of mRNAs resulting in protection against degradation enzymes, and in optimization of translation. Because TISU elements prevent leaky scanning and demonstrate strong initiation capacity, these 5′UTRs may be useful in the construction of exo mRNAs (see Subheading 2.1.1). Although each of these modifications separately may not be sufficient for the considerable increase of the duration of expression, due to the short lifetime of mRNA compared to protein, some combinations of these could be productive. Another part of such efforts would be the modulation of mRNA behavior outside translation, i.e., mRNA moving in and out of storage particles, cytoplasmic recapping and polyadenylation (See Subheadings 2.3 and 3).

Employment of new *cis* and *trans* regulatory elements for exo mRNA is an intriguing next step in exploring the method. Riboswitches and noncoding RNAs are powerful tools which could be adopted for that purpose . The riboswitches are RNA sensors that have been found in bacteria, fungi, and plants. They represent *cis*-elements that can control mRNA activity by binding small molecules, responding to ion concentration, temperature and other stimuli (189). These and similar elements could be designed for the introduction into the exo mRNAs in order to regulate exo mRNA activity in higher eukaryotes (190, 191). Noncoding RNAs, especially miRNAs are one of the central elements regulating mRNA metabolism (see Subheading 2). It is clear that transfer of combinations of RNAs that include coding and noncoding RNA molecules can target multiple metabolic pathways, and substantially increase the efficiency of cell reprogramming.

Improving existing methods and developing new methods for mRNA delivery will be important. This will probably include already known techniques for DNA and small RNA transfer, such as biolistics, jet injection, hydrodynamic injection, ultrasound, and magnetic field transfer (192). Finally, the greater safety of exo RNA than DNA may permit more rapid development of a range of therapies.

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Chapter 2

In Vitro Transcription of Long RNA Containing Modified Nucleosides

Norbert Pardi, Hiromi Muramatsu, Drew Weissman, and Katalin Karikó

Abstract

The in vitro synthesis of long RNA can be accomplished using phage RNA polymerase and template DNA. However, the in vitro synthesized RNA, unlike those transcribed in vivo in cells, lacks nucleoside modifications. Introducing modified nucleosides into in vitro transcripts is important because they reduce the potential of RNA to activate RNA sensors [1–6] and translation of such nucleoside-modified RNA is increased in cell lines, primary cells, and after in vivo delivery [1, 3, 7–10]. Here, we describe the in vitro synthesis of nucleoside-modified RNA with enhanced translational capacity and reduced ability to activate immune sensors.

Key words: messenger RNA, Immunogenicity, Modified nucleoside, Pseudouridine, 5-methylcytidine, Capping, In vitro transcription

1. Introduction

The in vitro delivery of protein-encoding DNA by transfection was first reported in 1973 (11) and 5 years later, a similar delivery of a protein-encoding viral RNA was also described (12). Over the subsequent years, DNA delivery became a standard laboratory technique, while RNA transfection was limited to viral RNA isolates until 1984 when in vitro transcription utilizing phage RNA polymerase and cloned cDNA was introduced (13). Interestingly, the first in vivo application of an in vitro transcript encoding a physiologically relevant protein was reported in 1992 (14), but RNAs further development beyond use as antigen-encoding vaccine vector (15–17) was delayed until 2011 (9). The potential reasons for the limited interest in applying mRNA as a therapeutic or using it in laboratory techniques for cellular delivery are RNA's immunogenicity, lability, low level and transient translatability, and

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difficulty to work with. The recent discovery that incorporating naturally occurring modified nucleosides into RNA reduces its immunogenicity and greatly increases its translatability (3, 4, 6–8, 10, 18) has revolutionized RNA as a therapeutic. Now, in vitro transcripts containing modified nucleosides have been tested in vitro, to express protein at a high efficiency (3, 7, 19); ex vivo, to deliver transcription factors for the generation of induced pluripotent stem (iPS) cells (20–22); and recently, in vivo to express therapeutic proteins in mice and macaques (9, 10, 14).

Generally, eukaryotic mRNAs contain protein-coding sequences flanked by 5′ and 3′ untranslated regions (UTRs) and 5′ cap and 3′ poly(A) tails at their extremities. One way to increase or regulate the half-life and translational efficiency of an in vitro transcribed mRNA is to optimize its UTRs. Favorable 5′ and 3′ UTRs can be obtained from cellular or viral RNAs. We have found that using a 5′ UTR derived from the tobacco etch virus 5′ leader RNA, which mediates cap-independent translation (23–26), results in rapid and high level translation of the coding sequence (3, 7, 10, 19). Similarly, applying the 3′ UTR of *Xenopus* beta-globin mRNA also ensures enhanced translation (27). To create an RNA expression plasmid, the coding sequence is cloned downstream from the promoter of T3, T7, or Sp6 RNA polymerase and flanked by 5′ and 3′ UTRs of interest. Sequences corresponding to a poly(A) tail can also be encoded. Incorporation of unique restriction enzyme recognition sites downstream of the poly(A) tail-related sequence aids in complete linearization of the plasmid. Linear DNA can also be prepared by PCR using primers containing the appropriate phage RNA polymerase promoter, but the choice of UTRs and encoded poly(A) tails is more limited. In addition, we found that RNA transcribed from PCR template contains more aberrant products.

After in vitro transcription, the newly synthesized mRNA can be further optimized. 7-methylguanylate cap and a poly(A) tail give significant stability and translatability to mRNA. Both can be incorporated into RNA during transcription with the inclusion of an anti-reverse cap analog (ARCA) and by encoding an extended region of polyadenylate, but these approaches have limitations. The use of ARCA reduces the amount of transcribed RNA per reaction, typically up to 50%, and a portion of the RNA remains uncapped and contains a 5′-triphosphate. The length of polyadenylate regions that a plasmid can tolerate without recombination is also limited. Vaccinia virus capping enzyme and 2′-O-methyltransferase (28) modify the 5′-triphosphate end of RNA with a cap structure containing terminal 7-methyl guanosine and 2′-O-methyl group at the penultimate nucleotide of the 5′-end (cap1). If the template plasmid or PCR product does not contain a region coding for poly(A) or the short poly(A) tail of the newly synthetised mRNA requires lengthening, poly(A) tail can be added to the 3′ end of the mRNA using yeast poly(A) polymerase.

Different nucleoside modifications can be incorporated into mRNA. We found that T7 RNA polymerase or the double mutant (Y639F/H784A) with superior ability to incorporate noncanonical NTPs (29) were able to incorporate 2-thiouridine (s2U), 5-methyluridine (m5U), 5-methylcytidine (m5C), N6-methyladenosine (m6A), and pseudouridine (Ψ), but were unable to incorporate 2′-O-methylated NTPs into long RNA (6). Other groups have incorporated modified nucleosides that are not naturally found in RNA (30, 31), however these RNAs are translated very poorly or not at all (32). In addition, we believe that the risk of using nonnatural nucleosides is high, due to their metabolism and ability to be reincorporated into cellular or mitochondrial DNA or RNA. We have found that for translation, complete replacement of uridine with pseudouridine results in RNA with the highest level of translation and low levels of immunogenicity. Incorporation of m5C and Ψ into the RNA results in a further decrease in immunogenicity and variable but usually lower levels of translation. The use of HPLC purification further decreases residual immunogenicity from both types of modified mRNA with mRNA containing Ψ resulting in the highest levels of translation with reduced cost (7). Partial replacement of one or multiple nucleosides has been studied, but immunogenicity of the RNA remains (6, 33).

2. Materials

Prepare all the solutions using RNase-free reagents. Store all reagents at −20°C unless otherwise indicated. Follow all waste disposal regulations when disposing waste materials.

2.1. Analysis of DNA and RNA by Non-denaturing Agarose Gel Electrophoresis

1. Sub-Cell GT Agarose Gel Electrophoresis System with 7 × 10 cm tray and 15-well 0.75 mm thick combs (Cat. number 170–4487) (Bio-Rad, Hercules, CA).
2. Sub-Cell GT/PowerPac Basic System (Cat. number 164-0302) (Bio-Rad).
3. Agarose for molecular biological applications, store at 4°C.
4. Lithium boric acid electrophoresis buffer (LB) (Cat. number #LB10-1) (Faster Better Media, Hunt Valley, MD), store at room temperature (RT).
5. 5× LB sample-loading media with orange G (Cat. number #LB5N-8) (Faster Better Media), store at RT.
6. Ethidium bromide (EtBr) stock: 0.5 mg/ml EtBr, store at RT.
7. UV transilluminator, we use a GelDoc 1000 gel imaging system (Bio-Rad).

2.2. Plasmid Linearization

1. Buffer and restriction enzymes that cleave at the 3′ end of desired RNA sequence, if using a plasmid as the template.
2. Phenol–chloroform–isoamyl alcohol (25:24:1), pH 8.0, store at 4°C.
3. Chloroform, store at RT.
4. NaOAc: 3.0 M NaOAc, pH 5.5, store at 4°C.
5. Isopropanol, store at –20°C.
6. Ethanol: 75%, store at 4°C.

2.3. In Vitro RNA Synthesis

1. In vitro transcription kits with or without modified nucleoside triphosphates and Anti-Reverse Cap Analog (ARCA). Kits provide the nucleoside triphosphate solutions, reaction buffer, DNase, and RNA polymerase enzyme (see Note 1).
2. Modified nucleoside triphosphates (NTPs) can be obtained separately for transcription kits that do not include them (see Note 2).
3. DNase- and RNase-free, ultrapure (UP) water, stored at RT.
4. Lithium chloride precipitation solution: 7.5 M LiCl, 50 mM EDTA.
5. Siliconized microcentrifuge tubes.
6. NanoVue (Cat. number 28-9569-62) (GE Healthcare Biosciences, Piscataway, NJ) or similar UV spectrophotometer for determination of RNA and DNA concentrations.

2.4. Cap Modification of In Vitro Transcribed RNA

1. ScriptCap m^{7}G Capping System (Cat. number C-SCCE0610) (CellScript, Madison, WI).
2. 2′-O-Methyltransferase kit (Cat. number C-SCMT0610) (CellScript).

2.5. Polyadenylation of RNA

1. Yeast poly(A) polymerase (600 U/μl, Cat. number 74225Y) (USB, Cleveland, OH) provided with 5× reaction buffer.
2. ATP stock: 100 mM ATP.

2.6. Dephosphorylation of RNA

1. APex heat-labile phosphatase provided with 10× buffer, pH 7.8 (Cat. Number APAP4850) (Epicentre, Madison, WI).
2. Acetic acid: 1 M, to adjust the pH of the reaction buffer, store at RT.

3. Methods

Plasmids constructed to contain the protein-coding sequence of interest, flanked by sequences corresponding to 5′ and 3′ UTRs, and encoded poly(A) tail are linearized prior to in vitro transcription (see Note 3). The RNA can be capped during transcription by incorporating an Anti-Reverse Cap Analog (ARCA) or after transcription using capping enzymes. If ARCA is incorporated during transcription, the ratio of GTP to ARCA can be varied to increase the percent of RNA transcripts containing cap, but this is done at the expense of the total amount of RNA made in the reaction. Transcription kits with ARCA use a ratio of ARCA to GTP of 4:1, which results in approximately 80% of the transcripts capped and a 50% reduction in the total amount of RNA made. If immune activation is a concern, ARCA-containing RNA can be treated with a phosphatase to remove 5′-triphosphates. A number of factors weight into the decision to cap transcriptionally versus after transcription. We have found that RNA with the highest level of translation and the least overall cost, especially if phosphatase treatment will be used, is generated with posttranscriptional capping. A long poly(A) tail can be added to the 3′ end of the RNA to further increase its stability and translatability using poly(A) polymerase.

All work with RNA is compromised by the presence of RNases. A major source of RNase contamination in the laboratory is plasmid DNA purification kits most of which use RNase A to remove bacterial RNA. RNase A renatures after drying or autoclaving, and therefore, special decontaminating procedures are needed. Ideally, a closed area isolated from reagents used for plasmid isolation should be secured for RNA synthesis.

3.1. Analysis of DNA and RNA by Non-denaturing Agarose Gel Electrophoresis

Standard agarose gel electrophoresis with EtBr staining is used to analyze RNA and DNA. Care to avoid RNase contamination of equipment or solutions should be taken.

1. Heat 0.5 g of agarose in 50 ml of 1× LB in a microwave until just boiling. This will make a 1% gel that is good for DNA and RNA above 0.5 kb in length. Higher concentrations of agarose can be used, when needed.
2. Cool to approximately 60°C, add 2 μl of EtBr stock solution, pour into a 7 cm × 10 cm gel tray, and add 1 or 2 15-well combs (0.75 mm thick).
3. Cool to room temperature, remove combs, and place gel tray into an electrophoresis apparatus containing 1× LB.
4. Apply 0.2–0.4 μg of DNA or RNA in 6 μl of 1× LB sample-loading media per well (see Note 4).
5. Apply a standard DNA or RNA ladder, if desired. RNA isolated from human cells can be used. Human 28S and 18S rRNAs are 5.0 and 1.9 kb, respectively.
6. Perform electrophoresis at 10 V/cm.
7. View migration of the nucleic acid intermittently by UV illumination (see Note 4).

3.2. Preparation of Linearized Plasmid DNA for In Vitro Transcription

In vitro RNA synthesis is performed on linear template DNA, preferentially from plasmids rather than PCR products. Template plasmid is linearized using a unique restriction endonuclease that leaves a 5′-overhang (preferred) or blunt end close to the encoded poly(A) sequence, if present (see Note 3). Restriction enzymes that leave a 3′ overhang should not be used, as this can result in the polymerase continuing to transcribe off of the opposite strand resulting in double stranded RNAs (34).

1. Digest 50 µg of plasmid DNA with 50–100 units of restriction enzyme(s) in a 100 µl reaction for 4 h or overnight. Use high fidelity restriction endonucleases, if possible, to avoid star activity. Use two enzymes that cleave a sufficient distance apart to allow both to cleave the same plasmid, if possible, to ensure complete cleavage of the plasmid (see Note 5).
2. Analyze by agarose gel electrophoresis with EtBr staining and UV illumination to confirm complete cleavage of the plasmid DNA (Fig. 1).
3. Isolate the linearized plasmid DNA by extracting with 50 µl of phenol–chloroform–isoamyl alcohol following standard techniques.
4. Re-extract the aqueous phase, which contains the linearized plasmid, with chloroform (see Note 6).
5. Precipitate the DNA with 1/10 volume of NaOAc and an equal volume of isopropanol.
6. Incubate at −20°C for at least 30 min.
7. Pellet the precipitated DNA for 2 min at 13,000 × *g* in a microcentrifuge at RT.
8. Wash three times with cold 75% ethanol (see Note 7).

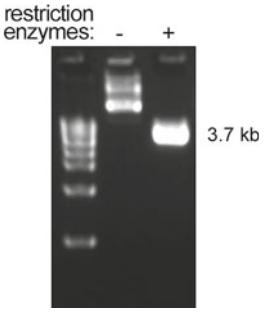

Fig. 1. Linearization of plasmid DNA. Aliquots of reaction mixtures incubated with or without restriction enzymes were separated on a 1% agarose gel. The ethidium bromide-stained bands are visualized after UV illumination. Although equal amounts of plasmids were loaded onto the gel, linearized plasmid, which is more accessible to ethidium bromide than circular plasmid, generates a stronger signal. A 1 kb DNA ladder (first lane) is used as a molecular weight reference.

9. Let the remaining ethanol evaporate by leaving the tube on the bench with the cap open for about 5 min.
10. Resuspended in UP water (see Note 8).
11. Measure the concentration of the linearized DNA using a spectrophotometer and reading the absorbance at 260 nm. The concentration is calculated by multiplying the OD260 by 0.05 to obtain μg/μl of DNA.

3.3. In Vitro RNA Synthesis

In vitro transcription kits are designed to produce high yields of RNA from linearized plasmid DNA template containing a T3, T7, or Sp6 promoter (see Note 9). One microgram of DNA template is sufficient to generate 50–100 μg of RNA.

1. For generating modified nucleoside-containing RNA with transcription kits not containing a modified nucleoside(s), exchange the basic NTP with the corresponding triphosphate-derivative of the modified nucleoside at the same concentration in the transcription reaction (see Note 10).
2. In a siliconized microcentrifuge tube for a 10 μl reaction volume, add 1 μg template DNA, reaction buffer, nucleoside triphosphate stocks (see Note 11), and RNA polymerase enzyme, per the kit being used.
3. Incubate the in vitro transcription reaction for 4 h at 37°C (see Note 12).
4. Degrade the DNA template with DNase by incubating at 37°C for 15 min.
5. Precipitate the RNA by adding ½ volume (5 μl) of cold LiCl precipitation solution.
6. Incubate overnight at –20°C.
7. Centrifuge the precipitated RNA at 13,000 × *g* for 5 min in a microcentrifuge at RT.
8. Wash the firm RNA pellet three times with cold 75% ethanol (see Note 7).
9. Let the remaining ethanol evaporate by leaving the tube on the bench with the cap open for about 5 min. Do not let the RNA pellet completely dry.
10. Resuspend the RNA pellet in UP water.
11. Freeze-thaw the dissolved RNA by placing the tube on dry ice until frozen and then on the bench at RT until thawed. Vortex the sample vigorously and repeat the freeze-thawing two more times to ensure complete rehydration of the sample. A –80°C or –20°C freezer can also be used, but freezing takes more time.
12. Measure the RNA concentration with a NanoVue.
13. Analyze the quality of the RNA by agarose gel electrophoresis and EtBr staining and UV illumination (see Note 4).

3.4. Posttranscriptional Capping of In Vitro Transcribed RNA

RNA can be capped posttranscriptionally using an enzyme derived from vaccinia virus, which forms cap0. The addition of a second enzyme that methylates the 2′-OH of the penultimate nucleotide at the 5′-end of the RNA produces cap1. The ScriptCap m^{7}G capping enzyme and 2′-O-methyltransferase (CellScript) are used. The enzymes can be used simultaneously in one reaction (see Note 13). Optimizing the 5′ sequence of the RNA to avoid formation of a stem-loop structure ensures that the capping enzyme has full access to convert all of the 5′ triphosphate ends (3), thereby capping all the RNA.

1. Add 60 μg of in vitro transcribed RNA (see Note 14) and UP water to a siliconized microcentrifuge tube and incubate at 65°C for 5 min to disrupt secondary structures of the RNA.
2. After heating, snap-cool the RNA on ice and add the remaining components of the capping reaction, 10 μl 10× reaction buffer, 10 μl GTP, 5 μl SAM, 2.5 μl ScriptGuard RNase inhibitor, 5 μl ScriptCap m^{7}G capping enzyme, and 5 μl 2′-O-methyltransferase in a 100 μl final volume.
3. Incubate at 37°C for 1 h.
4. Precipitate the RNA with 50 μl of cold LiCl precipitation solution.
5. Incubate at −20°C overnight.
6. Centrifuge the precipitated RNA at 13,000 ×*g* for 5 min in a microcentrifuge at RT.
7. Wash the firm RNA pellet three times with cold 75% ethanol (see Note 7).
8. Let the remaining ethanol evaporate by leaving the tube on the bench with the cap open for about 5 min. Do not let the RNA pellet completely dry.
9. Resuspend the RNA pellet in UP water.
10. Freeze-thaw the dissolved RNA by placing the tube on dry ice until frozen and then on the bench at RT until thawed. Vortex the sample vigorously and repeat the freeze-thawing two more times to ensure complete rehydration of the sample. A −80°C or −20°C freezer can also be used, but freezing takes more time.
11. Measure the concentration of the RNA with a NanoVue. The concentration is calculated by multiplying the OD260 by 0.04 to obtain μg/μl of RNA.
12. Analyze the quality of the RNA by agarose gel electrophoresis and EtBr staining and UV illumination (see Note 4).

3.5. Polyadenylation of In Vitro Transcribed RNA

Poly(A) tails can be encoded in the DNA used to transcribe RNA and/or added posttranscriptionally.

1. In a siliconized microcentrifuge tube for a 25 μl reaction, add 33 pmol of RNA, which correspond to ~10 μg of a 1 kb-long RNA (see Note 15).
2. Add 1.3 μl ATP stock, 5 μl 5× reaction buffer, and 2.5 μl poly(A) polymerase enzyme.
3. Incubate for 3 h at 37°C.
4. Precipitate RNA by adding 12.5 μl of cold LiCl precipitation solution.
5. Centrifuge the precipitated RNA at 13,000 × *g* for 5 min in a microcentrifuge at RT.
6. Wash the RNA pellet three times with cold 75% ethanol (see Note 7).
7. Let the remaining ethanol evaporate by leaving the tube on the bench with the cap open for about 5 min. Do not let the RNA pellet completely dry.
8. Resuspend the RNA pellet in UP water.
9. Freeze-thaw the dissolved RNA by placing the tube on dry ice until frozen and then on the bench at RT until thawed. Vortex the sample vigorously and repeat the freeze-thawing two more times to ensure complete rehydration of the sample. A −80°C or −20°C freezer can also be used, but freezing takes more time.
10. Measure the concentration of the RNA with a NanoVue.
11. Analyze the RNA by agarose gel electrophoresis and EtBr staining and UV illumination (see Note 4). Poly(A)-tailed RNA migrates slower relative to the RNA without poly(A) tail addition (see Note 16 and Fig. 2).

3.6. Removal of 5′-Triphosphate with APex Phosphatase

Most phosphatases are active only at ~pH 8 or higher. Considering that RNA is quickly hydrolyzed at such a high pH, removing 5′-end phosphates from RNA should be performed with APex alkaline phosphatase that is active at pH's as low as 5. APex phosphatase is provided with 10× acetate buffer (pH 7.5), but our experience is that RNA is still hydrolyzed in this buffer. Therefore, use acetic acid and UP water to convert the pH 7.5 acetate buffer (10×) to pH 6.5 buffer (5×).

1. Adjust the pH of the 10× buffer to 6.5 using 1 M acetic acid and a pH meter or narrow range (4 to 7) pH test strips.
2. Increase the final volume of the pH adjusted buffer to twice the starting volume to make a 5× buffer.

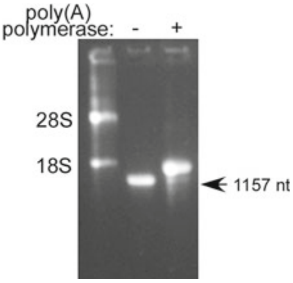

Fig. 2. Poly(A)-tailing of RNA. Aliquots of RNA before and after a 3 h incubation with poly(A) polymerase were separated on a 1.4% agarose gel. The ethidium bromide-stained bands are visualized after UV illumination. RNA isolated from human cells is used as a molecular weight reference. Human 28S and 18S rRNAs are 5.0 and 1.9 kb, respectively. A higher amount of poly(A)-tailed RNA was loaded to the gel to detect any RNA that remained unextended.

3. For a 50 μl reaction, add 10 μl of 5× acetate buffer (pH 6.5) to 25 μl of RNA (50 μg).
4. Add 15 μl of APex alkaline phosphatase (1 unit per μg of RNA).
5. Incubate at 37°C for 60 min.
6. Heat-inactivate by incubating at 70°C for 5 min.
7. Precipitate RNA by adding 25 μl (½ volume) of cold LiCl precipitation solution.
8. Centrifuge the precipitated RNA at 13,000 × *g* for 5 min in a microcentrifuge at RT.
9. Wash the firm RNA pellet three times with cold 75% ethanol (see Note 7).
10. Let the remaining ethanol evaporate by leaving the tube on the bench with the cap open for about 5 min. Do not let the RNA pellet completely dry.
11. Resuspend the RNA pellet in UP water.
12. Freeze-thaw the dissolved RNA by placing the tube on dry ice until frozen and then on the bench at RT until thawed. Vortex the sample vigorously and repeat the freeze-thawing two more times to ensure complete rehydration of the sample. A −80°C or −20°C freezer can also be used, but freezing takes more time.
13. Measure the concentration of the RNA with a NanoVue.
14. Analyze the RNA by agarose gel electrophoresis and EtBr staining and UV illumination (see Note 4).

4. Notes

1. RNA transcription kits, including INCOGNITO kits from CellScript (Madison, WI) (T7 ± ARCA) with Ψ and m5C (Cat. numbers: C-ICTAMY110510 and C-ICTMY110510, respectively), T7 with Ψ (Cat. number: C-ICTY110510), SP6 with Ψ (Cat. number: C-ICSY110510) and kits without modified nucleosides; SP6-Scribe (Cat. number: C-AS3106), T7 mScript (Cat. number: C-MSC11610), and T7-Scribe (Cat. number: C-AS2607) (CellScript) and Megascript and mMESSAGE mMACHINE (T7, T3, and SP6) (Cat. numbers: AM1333, AM1338, and AM1339 and AM1334, AM1348, and AM1340, respectively) (Ambion, Grand Island, NY) can be used.
2. Triphosphate-derivatives of modified nucleosides, including m5C (Cat. number N-1014), m5U (Cat. number N-1024), s2U (Cat. number N-1032), m6A (Cat. number N-1013), and Ψ (Cat. number N-1019) (TriLink, San Diego, CA) are prepared at a concentration of 50 mM (Sp6) or 75 mM (T3 or T7).
3. For plasmid linearization, restriction enzymes generating 5′-overhangs should be selected to avoid aberrant transcription leading to dsRNA production. To achieve 100% plasmid linearization, two restriction enzymes should be used that cut close (10–20 nucleotides) to each other.
4. Such a small amount of RNA can only be detected when non-denaturing agarose gel electrophoresis is used for the analysis. Under non-denaturing conditions, the RNA migration can be anomalous due to *cis*- and *trans*-molecular interactions, and thus, additional slower migrating bands might appear. Modified nucleoside-containing RNAs may migrate differently from the corresponding RNA that does not contain modified nucleosides. However, the bands should appear sharp and intact with no smear, a sign of RNA degradation. RNAs can also be analyzed under denaturing conditions to determine their correct length.
5. Incomplete plasmid linearization leaves contaminating circular plasmid templates that will be transcribed into extremely long and heterogeneous RNA due to the processivity of the RNA polymerases. Transcription initiation is a limiting step, thus, the presence of a small amount of circular template will lead to generation of a large amount of unwanted transcripts.
6. A chloroform extraction is performed to remove residual phenol that inhibits transcription.
7. The ethanol wash is performed by adding 100 μl of 75% ethanol per 10 μl of reaction volume, dislodging the pellet by using the force of adding the liquid, briefly centrifuging the tube at

high speed, and removing the liquid. Care should be taken when removing liquid, we do not use a vacuum aspirator but prefer a micropipettor, so that if the pellet is aspirated, it can be spun again. The siliconized tube allows the RNA pellet to be slightly less adherent, but the force of expelling the ethanol from a micropipette is needed to dislodge. Avoid breaking the pellet into pieces.

8. Typically, a third of the starting amount of DNA is lost during precipitation. The volume of water used to dissolve the DNA should aim to give a DNA concentration of 1–2 μg/μl.
9. The yields of mRNA are lower when transcription is performed with Sp6 RNA polymerase.
10. For RNAs that will contain only a fraction of modified nucleosides, the relative amounts of certain modified and its cognate unmodified NTP can be added to the transcription reaction. We found that for m5C, m6A, and Ψ, the percent of modified nucleotide in the transcription reaction equaled the percent incorporated into the RNA (6). Other modified nucleotides will have to be examined for efficiency of incorporation.
11. Thaw NTP stocks that will be used repeatedly on ice, thus avoiding their hydrolysis to NDP and NMP.
12. The length of the transcription reaction is typically 4 h, but can be optimized for each RNA, however, extended overnight incubation is not recommended because at low NTP concentrations, the T7 RNA polymerase exerts RNase activity (35).
13. Reactions can be scaled up or down depending on how much RNA is needed. We typically do not process more than 200 μl of a reaction in a 1.5 ml Eppendorf tube because of the difficulty of rehydrating the large thick RNA pellet.
14. RNA transcripts can form stable secondary structures involving the 5′-most nucleotides that can severely limit access by the capping enzyme. Heating followed by snap-cooling can partially disrupt the RNA secondary structure resulting in increased capping efficiency. Properly designed sequences in plasmid templates that avoid stem-loop formation at the 5′ end of RNA allow access for the capping enzyme, thus ensuring generation of completely capped RNA that can be measured by the loss of γ-^{32}P label from the RNA (3).
15. The concentration of ends of RNA is used to determine how much RNA can be efficiently poly(A) tailed. We have found that with this poly(A) tailing kit, 33 pmol of RNA can be nearly 100% poly(A) tailed. When calculating molarity for a 1,000 nt long RNA, ~0.32 μg RNA equals 1 pmol.
16. After poly(A)-tailing, the RNA length is increased significantly (~200 nt) that can be detected by analyzing RNA samples before

and after poly(A)-tailing with agarose gel electrophoresis. A lack of an increase in RNA length or an easily detectable amount of RNA with identical length to untailed RNA can be caused by the inclusion of too many RNA ends (>33 pmol) or the formation of secondary structures at the 3′end of the RNA that obstruct access of the poly(A) polymerase.

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Chapter 3

HPLC Purification of In Vitro Transcribed Long RNA

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Abstract

In vitro transcription of DNA with phage RNA polymerases is currently the most efficient method to produce long sequence-specific RNA. While the reaction can yield large quantities of RNA, it contains impurities due to various unwanted activities of the polymerases. Here, we described an easily performed HPLC purification that removes multiple contaminants from in vitro transcribed RNA and is scalable. The purified RNA is translated at much greater levels, especially in primary cells and in vivo. HPLC purification of RNA containing modified nucleosides that suppress RNA-mediated activation of innate immune sensors leads to a non-immunogenic RNA with superior translational capacity.

Key words: RNA, In vitro transcription, Nucleoside modification, HPLC

1. Introduction

The first in vivo delivery of mRNA encoding a therapeutic protein was reported in 1992 (1), but only recently has the delivery of mRNA for scientific and therapeutic purposes gained expanded interest. Potential uses include; delivery of mRNA encoding transcription factors to generate induced pluripotent stem (iPS) cells (2–4), in vivo administration to express therapeutic proteins (1, 5, 6), ex vivo delivery to expanded cells as a cancer therapeutic (7–11), as the vector for vaccines (12–14), and in vitro delivery to express protein at a high efficiency (15–17). The recognition that the immunogenicity of RNA could be reduced by the incorporation of modified nucleosides with an associated increase in translation (16, 18) potentially allows efficient expression of proteins in vivo and ex vivo without activation of innate immune receptors. Unfortunately, modified nucleoside-containing RNA transcribed by phage RNA polymerases still retains a low level of activation of such pathways (4, 16, 19, 20). This remaining activation of RNA sensors could be due to modified nucleosides that do not completely

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suppress the RNAs ability to activate sensors (5) or dsRNA contaminants that activate even in the presence of nucleoside modification (15). It is well established that RNA transcribed in vitro by phage polymerase contains multiple aberrant RNA, including short RNAs as a result of abortive transcription initiation events (21) and double stranded (ds)RNAs generated by RNA-dependent RNA polymerase activity (22), RNA-primed transcription from RNA templates (23), and self-complementary 3′ extension (24).

Multiple methods of purification from transcription or associated reactions (capping and poly(A)-tailing) to produce efficiently translated mRNA have been described, including precipitation either with alcohol (isopropanol, ethanol) in the presence of monovalent cations or with high concentration of LiCl that are supplied in commercial in vitro transcription kits, size exclusion columns, and matrices based on silica and other compounds that bind RNA. Each of these procedures removes free nucleotides, proteins, salt, and short RNA oligos with varying efficiencies, but none are capable of removing long RNA contaminants or contaminants bound to the RNA of interest. Preparative denaturing polyacrylamide gel electrophoresis is commonly used to purify in vitro transcribed RNA; however, this method is suitable only for short RNAs (reviewed in ref. 25). Long RNAs can be separated on denaturing agarose gels, but they are not translatable due to covalent modifications introduced by the denaturants glyoxal and formaldehyde (26).

The continued development of mRNA as a research tool or for therapeutics requires the RNA to have high translatability, and in many instances, to avoid RNA sensor activation. The continued development of mRNA as a vector for vaccines, which is currently in human trials, and other therapeutic approaches also requires the highest levels of translation for both cost and potency. The HPLC approach described here allows efficient and scalable purification yielding RNA that can produce up to 1,000-fold more protein in primary cells and in vivo compared to unpurified in vitro transcribed RNA through the removal of dsRNA contaminants that activate RNA sensors that inhibit protein translation directly (reviewed in ref. 27, 28) or indirectly (reviewed in ref. 29). In addition, when such purified RNA contains certain modified nucleosides, it can also ablate activation of innate immune RNA sensors resulting in a highly translatable non-immunogenic RNA (15).

2. Materials

Use RNase-free and HPLC grade reagents, whenever possible. Acetonitrile is toxic, ensure proper protection is used. Its major toxicity is due to metabolism to hydrogen cyanide. The onset of toxic effects typically occurs 2–12 h after exposure, due to the requirement for the metabolism to cyanide. Follow all waste disposal regulations when disposing waste materials.

2.1. HPLC Purification

1. HPLC system capable of monitoring optical density at 260 nm fitted with a fraction collector and a column heater. We use an ÄKTApurifier 10 FPLC with a Frac-920 fraction collector (GE Healthcare Biosciences, Piscataway, NJ) and a TL105 column heater (Timberline Instruments, Boulder, CO).
2. HPLC columns contain a nonporous matrix consisting of polystyrene-divinylbenzene (PS-DVB) copolymer beads (2 μm) alkylated with C-18 chains (Transgenomic, Omaha, NE) (see Note 1).
3. Buffer A: 0.1 M triethylammonium acetate (TEAA), pH 7.0 (Cat. number 553401) (Transgenomic).
4. Buffer B: 0.1 M TEAA, 25% acetonitrile, pH 7.0 (Cat. number 553402) (Transgenomic).
5. Acetonitrile: 100% for HPLC system cleaning.
6. NaOH: 0.1 M for HPLC system cleaning.
7. HPLC grade water.
8. Ethanol: 20% for long-term storage of HPLC system.

2.2. Concentration of RNA from Column Fractions

1. Amicon Ultra-15 centrifugal filter units (30K membrane) (Cat. number UFC903096) (Millipore, Billerica, MA).
2. Sorvall ST16R centrifuge (Thermo Scientific, Asheville, NC) or similar.
3. Nuclease-free water.
4. NaOAc: 3.0 M, pH 5.5.
5. Isopropyl alcohol.
6. Glycogen (Cat. number 10901393001) (Roche Applied Science, Indianapolis, IN) (see Note 2).

2.3. Analysis of RNA

2.3.1. dsRNA Dot Blot

1. Super charged Nytran membranes.
2. TBS-T buffer: 50 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4.
3. Blocking buffer: 5% nonfat dried milk in TBS-T buffer.
4. Incubation buffer: 1% nonfat dried milk in TBS-T buffer.
5. dsRNA-specific mAb J2 (English & Scientific Consulting, Szirák, Hungary).
6. dsRNA-specific mAb K1 (English & Scientific Consulting).
7. HRP-conjugated donkey anti-mouse IgG (Cat. number 715-035-150) (Jackson ImmunoResearch, West Grove, PA).

8. ECL Plus Western blot detection reagent (Cat. number RPN2109) (Amersham/GE Healthcare Biosciences).
9. Fujifilm LAS1000 digital imaging system or similar.

2.3.2. RNA Translation

1. TransIT-mRNA transfection reagent containing TransIT-mRNA transfection and boost reagents (Cat. number MIR 2225) (Mirus Bio, Madison, WI) (see Note 3).
2. Human monocyte derived dendritic cells (DCs) for measuring RNA translation and immunogenicity (see Note 4).
    (a) Plate human primary monocytes at 1×10^6/ml, 3 ml per well in a 6-well plate in Aim V medium (Cat. number 12055-091) (Invitrogen, Grand Island, NY) supplemented with 50 ng/ml GM-CSF (Cat. number 215-GM-050) and 100 ng/ml IL-4 (Cat. number 204-IL-050) (R&D Systems, Minneapolis, MN).
    (b) On day 3 and 6 of culture, remove 1 ml of media from each well and add 1.5 ml of fresh Aim V medium supplemented with 50 ng/ml GM-CSF and 100 ng/ml IL-4.
    (c) On day 7–9, collect non-adherent cells and plate in a 96-well flat bottom plate in RPMI medium with 10% FCS and glutamine for transfection (see Note 5).
3. Luciferase Cell Culture Lysis 5× Reagent (Cat. number E1531) (Promega, Madison, WI) (see Note 6).
4. Firefly luciferase assay buffer (Cat. number E1500) (Promega) (see Note 6).
5. Luminometer, LUMAT LB 950 (Berthold/EG&G, Wallac/PerkinElmer, Waltham, MA) or similar.

2.3.3. RNA Immunogenicity

1. DCs (5×10^4 cells/well) in 96-well plates (see Note 7).
2. Poly(I:C): 50 μg/ml as a positive control.
3. Human IFN-α ELISA assay.

3. Methods

HPLC purification of RNA can be performed before or after the posttranscriptional addition of a 5′ cap with or without methylation (ScriptCap m7G capping system and ScriptCap 2′-O-methyltransferase kit, CELLSCRIPT) (see Note 8) or addition of poly(A) tail with poly(A) polymerase. The in vitro transcription reaction with or without DNase treatment or associated posttranscriptional modification reactions can be directly added to the HPLC column or any standard method of initial purification can be used before application to the HPLC column.

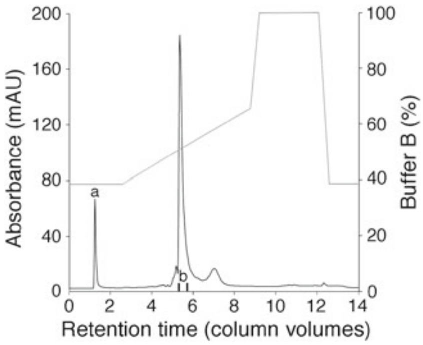

Fig. 1. HPLC purification of in vitro transcribed mRNA. RNA was applied to the HPLC column and eluted using a linear gradient of Buffer B (0.1 M TEAA, 25% acetonitrile, pH 7.0) in Buffer A (0.1 M TEAA, pH 7.0). The gradient spanned 38–65% Buffer B over 6 column void volumes (*gray line*). Absorbance at 260 nm was analyzed (*black line*), which demonstrated the expected sized RNA, as well as smaller and larger RNA species. A small peak is observed in the flow through (**a**). Column fraction selection noted by the *vertical black lines* on the *x*-axis (**b**) exclude RNA eluting before and after the expected major RNA peak.

Purify mRNA by LiCl sedimentation prior to HPLC.

The advantage of using a standard purification (LiCl or spin columns) prior to HPLC purification is that it helps to identify overloading of the HPLC column. When a standard purification is used first, a small peak measured by the OD at 260 nm representing residual nucleotides or other trace components of the transcription reaction will appear in the flow through (Fig. 1, noted as *a*). If the column is overloaded with RNA, a greater OD260 signal appears in the flow through fraction representing unbound RNA. In the absence of pre-purification, the flow through is overwhelmed by the constituents of the transcription reaction, namely nucleotide triphosphates, that give a signal 10–100-times greater than the purified RNA signal.

3.1. HPLC Purification of RNA Using a 35 ml Column

1. Set column oven to 45°C (see Note 9).
2. Set the flow rate to 5 ml/min.
3. Equilibrate column with 3 column void volumes (see Note 10) of 38% buffer B.
4. Load column with RNA in water (see Note 11) at one half the standard flow rate, 2.5 ml/min. Run 1.5 column void volumes of 38% buffer B after loading RNA.
5. Increase flow rate to 5 ml/min and run a 38–65% linear gradient of buffer B over 6 column void volumes.
6. Collect 5 ml column fractions corresponding to the major RNA peak excluding RNA eluting prior to and after (Fig. 1, noted as *b*).
7. Run a linear gradient from 65% to 100% buffer B over 0.2 column void volumes.

8. Run 100% buffer B for 3 column void volumes.
9. Run a linear gradient from 100% to 38% buffer B over 0.2 column void volumes.
10. Equilibrate with 38% buffer B for 1.5 column void volumes (see Note 12 for standard HPLC protocol).

3.2. RNA Contamination of HPLC Column

RNA contamination from previous purifications is found. For purifications where RNA immunogenicity is being reduced or the presence of contamination with the previously purified RNA is not acceptable, cleaning of the column and attention to the order that RNAs are purified is important. An unmodified RNA, which remains immunogenic after HPLC purification (15), should not be run prior to an RNA where no immunogenicity is required.

Run a cleaning program between RNA samples to reduce contamination.

1. Run 1 column void volume of 38% buffer B.
2. Increase to 100% buffer B over 0.2 column void volumes.
3. Run 1 column void volume of 100% buffer B.
4. Decrease to 38% buffer B over 0.2 column void volumes.
5. Repeat steps 1–4 two additional times for a total of 3 cycles (see Note 13 for HPLC cleaning protocol) (see Note 14).

3.3. HPLC Column and System Storage and Cleaning

Standard HPLC protocols for short and long term storage of the HPLC columns and system should be followed.

1. For short-term storage, less than 4 days, wash the column and system with 10 column void volumes of HPLC grade water.
2. For longer-term storage, wash the column and system with 10 column void volumes of 20% ethanol after the HPLC grade water wash.

Weekly HPLC cleaning or at the completion of purifications prior to an extended period of nonuse.

1. Clean the HPLC system with 100% acetonitrile for 10 column void volumes.
2. Clean the HPLC system with HPLC-grade water for 10 column void volumes.

Complete HPLC cleaning. When RNA with no contaminating RNA from previous runs is needed or when purification efficiency drops.

1. Clean the column and system with 10 column void volumes of 0.1 M NaOH.
2. Clean the column and system with 20 column void volumes of HPLC-grade water.

3. Extensively re-equilibration with 38% buffer B after NaOH cleaning, run 38% buffer B at 5 ml/min for 10 column void volumes.
4. Run 38% buffer B at 0.5 ml/min continuously for 3–4 days.

3.4. Purification of RNA from Column Fractions

1. Concentrate and desalt RNA from the desired column fractions in Amicon Ultra-15 centrifugal filter units (30K membrane).
2. Add column fractions to the filter.
3. Centrifuged at 4,000×*g* for 10 min (25°C) in a ST16R centrifuge.
4. Dilute concentrated RNA with nuclease-free water.
5. Repeat concentration and dilution (steps 1–4) two more times or until below a desired concentration of acetonitrile.
6. Recover RNA with overnight precipitation at −20°C by adding 1/10th volume NaOAc, 1 volume of isopropanol, and 3 μl of glycogen.

3.5. Analysis of RNA

3.5.1. dsRNA Dot Blot

A major contaminant that is found in all RNA sequences and with all modified nucleosides studied thus far is dsRNA. Binding of dsRNA-specific mAb J2 occurs even when the dsRNA contains modified nucleosides, e.g., pseudouridine and/or 5-methylcytidine, while binding of the other dsRNA-specific mAb K1 is reduced when dsRNA contains such modifications (15) (see Note 15).

1. Blot RNA (200 ng) onto super charged Nytran membranes and dry (see Note 16).
2. Block membranes with blocking buffer for 1 h.
3. Incubate membranes with dsRNA-specific mAb J2 or K1 (5,000-fold dilution) in incubation buffer for 60 min.
4. Rinse membranes four times and then wash six times (5 min for each wash) with TBS-T buffer.
5. React membranes with HRP-conjugated donkey anti-mouse IgG (5,000-fold dilution) in incubation buffer for 60 min.
6. Rinse membranes four times and then wash six times (5 min for each wash) with TBS-T buffer.
7. Detect membranes with ECL Plus Western blot detection reagent.
8. Capture images on a Fujifilm LAS1000 or similar digital imaging system (see Note 17).

3.5.2. RNA Translation

1. Complex HPLC-purified and corresponding unpurified firefly luciferase encoding mRNA to TransIT-mRNA (see Note 3) according to the manufacturer by combining RNA (0.1 μg)

with 17 μl serum free RPMI and adding TransIT-mRNA transfection reagent (0.3 μl) and boost reagent (0.2 μl).

2. Add 17 μl of complexed RNA to a single well of DCs in a 96-well plate in 180 μl of RPMI 10% FCS medium (see Notes 4 and 5).
3. Three to 24 h later, remove medium and lyse cells in 25 μl of Luciferase Cell Culture Lysis Reagent (see Note 18).
4. Measure mRNA translation by adding 2 μl of cell lysate to 10 μl of Firefly luciferase assay buffer and measuring for 10 s in a luminometer (see Note 19).

3.5.3. RNA Immunogenicity

1. Treat DCs (5×10^4 cells/well) in 96-well plates with medium, poly(I:C) (50 μg/ml) not complexed to TransIT transfection reagent (positive control) (see Note 20), and TransIT transfection reagent-complexed RNA (0.1 μg/well), as prepared above in RNA translation (Subheading 3.5.2).
2. Harvest supernatant after 24 h.
3. Assay for IFN-α by ELISA on undiluted supernatant.

4. Notes

1. A small column (7.8 mm diameter by 50 mm long (2.4 ml total volume)) can be directly obtained from Transgenomics (http://www.transgenomic.com/) (Cat # RNA-99-3810). It can purify up to 10 μg of RNA and runs at a flow rate of 1 ml/min. Larger columns can be special ordered from Transgenomic. We use a 21.2 mm diameter by 100 mm long column (35 ml total volume) that allows up to 600 μg of RNA per run to be purified.
2. Numerous commercially available glycogen preparations were found to be contaminated with RNA and DNA (30). We found that Roche Applied Science glycogen was free of DNA or RNA contamination.
3. Other transfection reagents compatible with long RNA can be used.
4. Human DCs are optimal for analyzing mRNA as they are primary cells and contain all known RNA sensors (31) and their ability to translate exogenously delivered mRNA is very sensitive to contaminants. Other primary cells or cell lines can be used.
5. TransIT-mRNA transfection reagent requires serum in the medium of cells when they are transfected.

6. Translation can be measured for any encoding mRNA with the appropriate quantitative assay.
7. Other cell types or cell lines can be used for measuring RNA immune activation. The appropriate assay to measure immunogenicity is needed and can include IFN-β, TNF-α, or IL-8 ELISAs depending on the cell used.
8. If 5′-cap is added enzymatically after HPLC purification, ensure that the RNA is precipitated with ½ volume of LiCl without adding alcohol to avoid S-adenosyl-methionine contamination that can reduce translation.
9. RNA purification can be optimized for maximum translation per RNA molecule transfected and, if it contains nucleoside modifications, for lack of immunogenicity. Each RNA has an optimal temperature for purification that results in the highest level of translation. For optimal performance, this should be determined for each RNA. We have observed differences of up to a twofold increase in translation comparing purifications at different temperatures. It is typically in the 45–55°C range.
10. The void volume of an HPLC column is the total volume of the liquid or mobile phase in a packed column. It is typically 50% of the total column volume for alkylated (PS-DVB) copolymer beads.
11. The column can be loaded with RNA in the constituents of the transcription reaction or post transcription reactions (cap or poly(A) tail addition) without any purification. Run 3 column void volumes of 38% buffer B after loading RNA in this fashion.
12. HPLC program for purifying RNA using a 35 ml column:
    (a) Column Pressure Limit 25.00 MPa
    (b) Averaging Time UV 1.30
    (c) Flow Rate 5 ml/min
    (d) Start 38% Buffer B
    (e) Equilibrate with 3 column void volumes
    (f) Flow Rate 2.5 ml/min
    (g) Inject sample
    (h) Empty loop with 6 ml
    (i) Wash column with 3 column void volumes
    (j) Flow Rate 5 ml/min
    (k) Start fraction collection for mAU>20 (dependent on amount and type of RNA), 1–5 ml fractions
    (l) Linear gradient to 65% Buffer B over 6 column void volumes
    (m) Stop fraction collection

   (n) Linear gradient to 100% Buffer B over 0.2 column void volumes
   (o) Equilibrate with 100% Buffer B for 3 column void volumes
   (p) Linear gradient to 38% Buffer B over 0.2 column void volumes
   (q) Equilibrate with 38% Buffer B for 1.5 column void volumes

13. HPLC program for cleaning between runs using a 35 ml column:
   (a) Column Pressure Limit 25 MPa
   (b) Flow Rate 5 ml/min
   (c) Start 38% Buffer B
   (d) Equilibrate with 38% Buffer B for 1 column void volume
   (e) Linear gradient to 100% Buffer B over 0.2 column void volumes
   (f) Equilibrate with 100% Buffer B for 1 column void volume
   (g) Linear gradient to 38% Buffer B over 0.2 column void volumes
   (h) Equilibrate with 38% Buffer B for 1 column void volume
   (i) Linear gradient to 100% Buffer B over 0.2 column void volumes
   (j) Equilibrate with 100% Buffer B for 1 column void volume
   (k) Linear gradient to 38% Buffer B over 0.2 column void volumes
   (l) Equilibrate with 38% Buffer B for 1 column void volume
   (m) Linear gradient to 100% Buffer B over 0.2 column void volumes
   (n) Equilibrate with 100% Buffer B for 1 column void volume
   (o) Linear gradient to 38% Buffer B over 0.2 column void volumes
14. For additional column cleaning to reduce contamination by RNA from previous runs, a single cycle of the above cleaning cycle (see Note 13) can be run prior to equilibration with buffer B in the standard RNA purification run (see Note 12).
15. The effect of other nucleoside modifications on binding by the dsRNA-specific mAbs will need to be determined.
16. dsRNA (25 ng) can be used as a positive control.

17. Hybridization with labeled DNA complementary to the desired RNA can be performed to confirm RNA loading on the membrane.
18. Both translation and immunogenicity of RNA can be measured with TransIT-mRNA complexing at 24 h post transfection. The peak and duration of translation varies with other transfection reagents, which will have to be determined.
19. RNA encoding other proteins can be analyzed with the appropriate assay.
20. R-848 or other TLR7 or TLR7/8 agonists can be used as a positive control for DCs.

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Chapter 4

Synthetic mRNAs with Superior Translation and Stability Properties

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Abstract

The translational efficiency and stability of synthetic mRNA in both cultured cells and whole animals can be improved by incorporation of modified cap structures at the 5′-end. mRNAs are synthesized in vitro by a phage RNA polymerase transcribing a plasmid containing the mRNA sequence in the presence of all four NTPs plus a cap dinucleotide. Modifications in the cap dinucleotide at the 2′- or 3′-positions of m^7Guo, or modifications in the polyphosphate chain, can improve both translational efficiency and stability of the mRNA, thereby increasing the amount and duration of protein expression. In the context of RNA-based immunotherapy, the latter is especially important for antigen production and presentation by dendritic cells. Protocols are presented for synthesis of modified mRNAs, their introduction into cells and whole animals, and measurement of their translational efficiency and stability.

Key words: Boranophosphate, Cap analogs, Decapping, Gene therapy, Immunotherapy, mRNA stability, Nucleoporation, Phosphorothioate, Protein expression, Translational efficiency

1. Introduction

The ability to make mRNA in vitro has proven to be valuable for a number of applications, e.g., synthesis of proteins in cell-free translational systems, studying the mechanisms of mRNA processing, and understanding the factors that determine mRNA turnover (1–3). One obstacle that must be surmounted in the synthesis of mRNA, however, is incorporation of the 5′-terminal m^7GTP-containing cap. Two principle approaches have been taken. In the first, a polynucleotide chain is synthesized in vitro with an RNA

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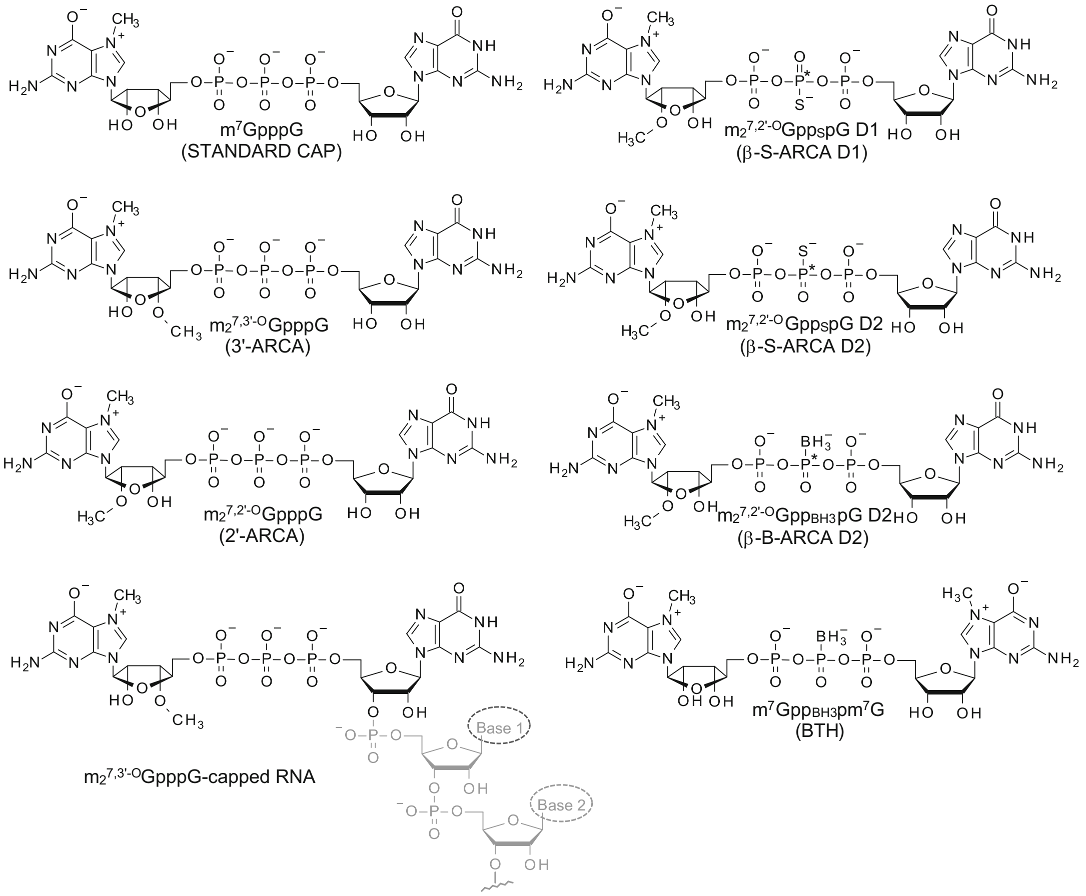

Fig. 1. Cap analog structures. The figure shows the cap dinucleotides used in the protocols described in this chapter as well as the 5′-terminal structure of mRNA into which the 3′-ARCA has been incorporated. D1 and D2 designate two diastereomers that differ only in the absolute configuration at the stereogenic β-phosphorus [marked with *asterisk* (*)]. The positions of S and BH_3 substituents in the figure do not refer to the absolute configurations (R_p and S_p) of D1 and D2 since these have not yet been determined.

polymerase, and then a complex of enzymes from vaccinia virus is used to incorporate GTP in a 5′-5′ triphosphate linkage, with concomitant production of P_i and PP_i, followed by 7-methylation of the 5′-terminal G with S-adenosylmethionine (4). In the second, RNA synthesis is carried out in the presence of all four NTPs plus a cap dinucleotide of the form m^7GpppG (Fig. 1) (1–3). The RNA polymerase incorporates the cap dinucleotide in place of GTP at the 5′-end of the RNA. To increase the percentage of capping, the concentration of GTP is lowered relative to the other NTPs and the concentration of cap dinucleotide is elevated.

Both methods are widely used because each has advantages and disadvantages. The vaccinia enzyme-mediated method yields a higher percentage of capping, but it is limited to synthesis of unmodified caps (or caps that are derived from GTP analogs that are recognized by the viral capping enzymes). The cap dinucleotide method can be used to synthesize mRNAs containing a wide

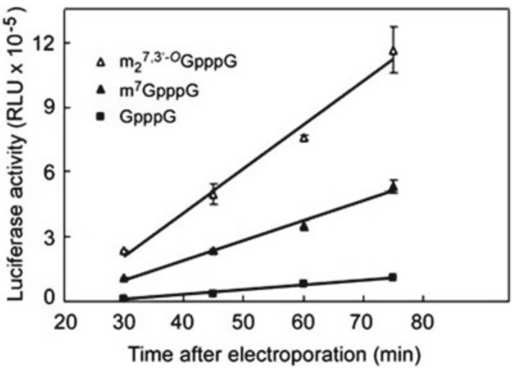

Fig. 2. Translational efficiency of modified mRNAs in cultured mammalian cells. mRNAs containing the standard cap versus the 3′-ARCA are compared. Luc-A_{60} mRNAs containing the indicated cap analogs were introduced into MM3MG cells by electroporation, which were then lysed at the indicated time points. Equal amounts of total protein were assayed for luciferase activity by luminometry. Relative light units (RLU) were normalized for the luciferase mRNA present in the cells 30 min after electroporation as measured by qRT-PCR. These data were originally published in Grudzien et al. (15) © the American Society for Biochemistry and Molecular Biology.

range of modified cap structures, but the capping efficiency is only about 90% under typical conditions. Another disadvantage of the latter method is that a cap dinucleotide such as m^7GpppG is incorporated in either orientation (5). This is because, during the initiation of RNA synthesis, the attack at the α-phosphate of the first nucleotide of the growing RNA chain can be made by the 3′-OH of either the Guo or the m^7Guo moiety of the cap dinucleotide. When attack is by the 3′-OH of the Guo moiety, a normal linkage results: m^7GpppGpG. When attack is by the 3′-OH of the m^7Guo moiety, a reversed linkage results: Gpppm^{7}GpG. Based on structural information of proteins involved in mRNA processing, translation, and turnover (6–8), the latter structure would not be recognized as a cap.

We (9, 10) and others (11–14) solved this problem by synthesizing cap dinucleotides that are incapable of being incorporated in the reverse orientation due to modifications at either the 2′ or 3′ positions of the m^7Guo moiety. We termed such analogs anti-reverse cap analogs (ARCAs), e.g., 2′-ARCA and 3′-ARCA in Fig. 1 (9). Use of such analogs produces mRNAs with superior translational properties both in vitro (9, 10, 15) and in cultured cells (15) (Fig. 2). An additional benefit of the unambiguous orientation is that it ensures precise location of a modification within the cap with respect to the mRNA body and, consequently, within the active sites of enzymes that recognize the cap. For instance, a modification of the phosphate proximal to the m^7Guo moiety will always be on the phosphate distal to the mRNA body, provided there is an ARCA modification to prevent reverse incorporation.

This latter feature proved to be useful after it was discovered that decapping is an early step in mRNA turnover (16). The yeast

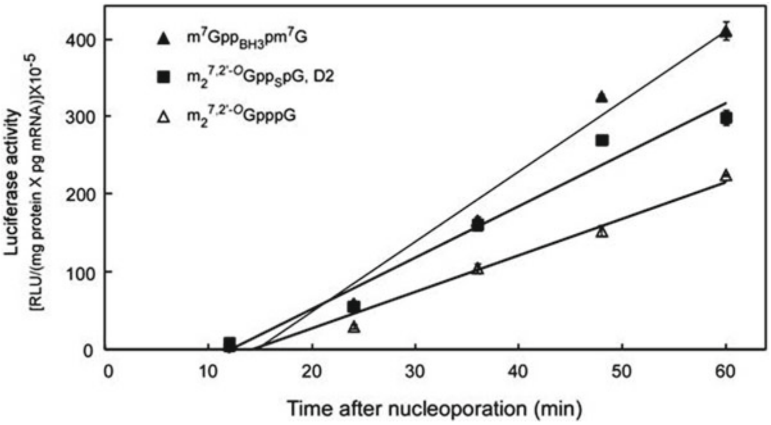

Fig. 3. Translational efficiency of modified mRNAs in cultured mammalian cells: mRNA containing the 2′-ARCA, β-S-ARCA D2, and BTH are compared. Luciferase activity was measured at various times after introduction of Luc-A_{60} mRNA capped with the indicated cap analogs into HeLa cells by nucleoporation. The data were normalized for the amount of luciferase mRNA as measured by qRT-PCR. These data were originally published in Su et al. (23) © 2011 RNA Society.

heterodimer Dcp1/Dcp2, and mammalian orthologs of Dcp2, cleave the cap between the α and β phosphates, liberating m^7GDP and a 5′-phosphate-terminal mRNA that is then a substrate for the 5′→3′ exonuclease Xrn1 (17–20). Substitution of the α-β bridging O atom with CH_2 in the cap dinucleotide to produce $m_2^{7,3'-O}GppCH_2pG$ prevents cleavage of this bond in vitro by the human ortholog of Dcp2, hDcp2, and stabilizes the mRNA to intracellular degradation in cultured cells (15). Unfortunately, mRNA capped with $m_2^{7,3'-O}GppCH_2pG$ is poorly translated in cultured cells because $m_2^{7,3'-O}GppCH_2pG$ has a lower affinity for the cap-binding protein eIF4E than the 3′-ARCA (15).

A different substitution of S for the non-bridging O on the β-phosphate to make the cap dinucleotide $m_2^{7,2'-O}Gpp_SpG$ (β-S-ARCA in Fig. 1) allowed us to synthesize mRNA that is resistant to in vitro decapping by hDcp2, more stable in cultured cells, and translated efficiently in cultured cells (21) (Figs. 3 and 4). Because this substitution creates a stereogenic β-phosphorus atom, two diastereomers of β-S-ARCA exist. Fortunately, these can be separated by HPLC, the two forms being termed D1 and D2 (Fig. 1) to signify their order of elution (22). β-S-ARCA D1 has a higher affinity for eIF4E than β-S-ARCA D2 (22), but mRNA capped with β-S-ARCA D2 is more efficiently translated in cultured mouse mammary epithelial cells (21). The reason for this apparent paradox is not known; one can speculate that translational initiation is impaired if the affinity of the cap for eIF4E is too high. On the other hand, mRNA capped with β-S-ARCA D2 is more slowly cleaved in vitro by hDcp2 than RNA capped with β-S-ARCA D1 (21). An unexpected surprise was that mRNA capped with β-S-ARCA D2 is translated 2.4-fold more efficiently in cultured cells

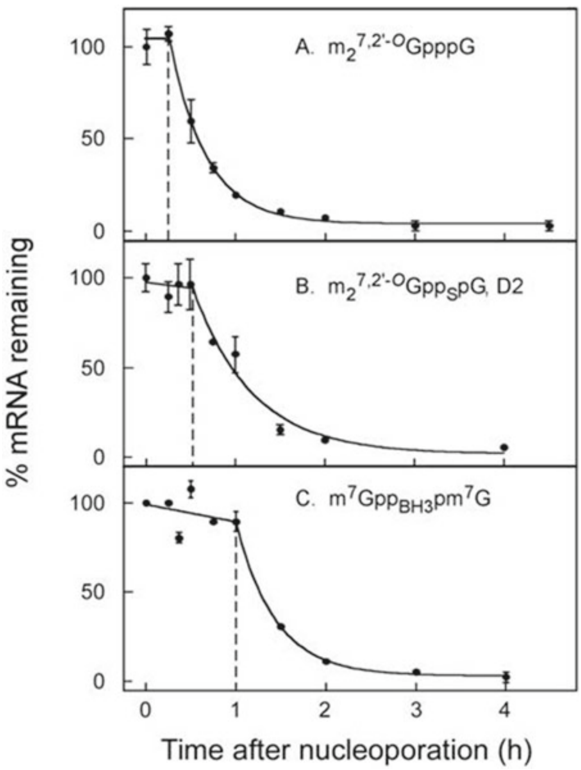

Fig. 4. Stability of modified mRNAs in cultured cells. Luc-A$_{60}$ mRNAs were synthesized in the presence of the indicated cap analogs and delivered into HeLa cells by nucleoporation. Cells were lysed at the indicated times and luciferase mRNA was measured by qRT-PCR. Data are plotted as a percentage of the luciferase mRNA present immediately after nucleoporation. In panels (**b**) and (**c**), there is a lag before the initiation of rapid decay. The data for the post-lag period were fit to single-exponential function and the $t_{1/2}$ of calculated as described in Subheading 3.5. *Vertical dashed lines* mark the boundary between lag phase and first-order decay phase. These data were originally published in Su et al. (23) © 2011 RNA Society.

than the same mRNA capped with the 2′-ARCA and 5.1-fold more efficiently than mRNA capped with m^{7}GpppG (21), even though we had explored the S-for-O substitution only in an attempt to confer resistance to cleavage by decapping enzymes.

Despite the fact that mRNA capped with β-S-ARCA D2 is resistant to cleavage by hDcp2 in vitro, it is still slowly cleaved (23). We therefore developed another series of cap analogs to address this problem. We found that substitution of the β non-bridging O with BH$_3$ to form m^{7}Gpp$_{BH3}$pm^{7}G (BTH in Fig. 1) yielded mRNA that was more resistant to cleavage by hDcp2 in vitro than mRNA capped with β-S-ARCA D2, yet it was still translated as efficiently in HeLa cells (23) (Fig. 3).

The modified mRNAs have superior properties not only in cultured cells but also in whole animals (24). Injection of luciferase mRNA capped with β-S-ARCA D1 into the lymph nodes of mice, where the RNA is taken up by immature dendritic cells (25),

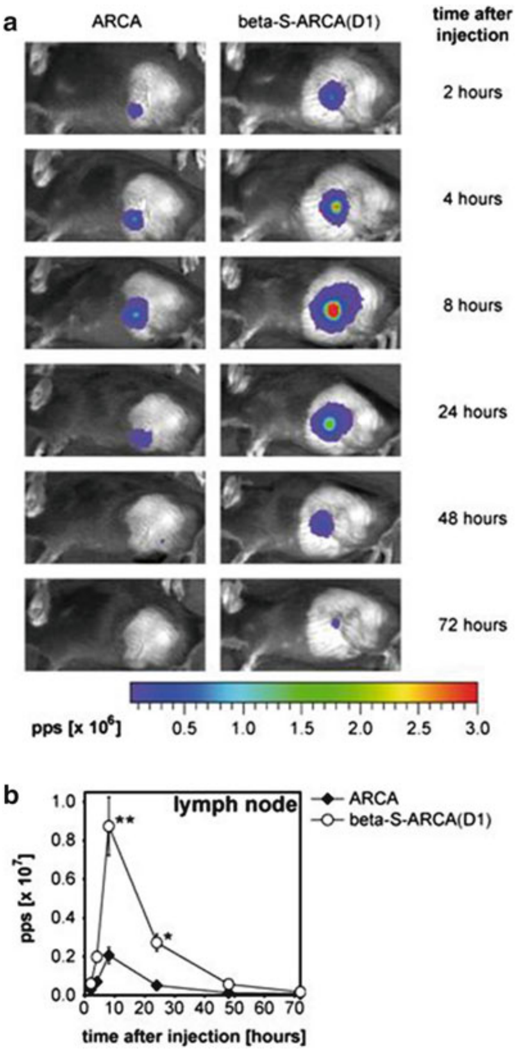

Fig. 5. Enhanced production of proteins by modified mRNAs in whole animals. Two groups of mice (each $n = 9$) received injections of equal amounts of luciferase-encoding mRNA capped with either the 2′-ARCA or β-S ARCA D1 into inguinal lymph nodes. In vivo imaging of luciferase activity (given in pps) was performed at different time points. (**a**) Imaging series of representative mice. The photon counts are represented as given by the scale at the bottom. (**b**) Kinetics of average luciferase activity. These data were originally published in Kuhn et al. (24) © 2010 Macmillan Publishers Limited.

produces more luciferase than the same ARCA-capped mRNA (Fig. 5). Interestingly, in this case the D1 isomer of β-S-ARCA provides a greater response than the D2 isomer, as also observed in cultured immature dendritic cells (24), but the opposite of what was observed in cultured mammary epithelial cells (21). The reasons for these differences are unclear.

In this chapter, we provide protocols for each stage of the work described above: (1) construction of a plasmid template for synthesis of an mRNA containing a 50-nt poly(A) tail with no additional 3′-terminal nucleotides, (2) in vitro synthesis of mRNA containing modified caps from this template, (3) introduction of synthetic mRNAs into cultured mammalian cells, (4) analysis of translational efficiency in cultured cells, (5) analysis of mRNA stability in cultured cells, and (6) production of proteins in experimental animals with synthetic mRNAs.

2. Materials

2.1. Cap Analogs

Cap analogs that have been used to synthesize mRNAs with superior translational efficiency and stability are shown in Fig. 1.

1. m^7GpppG. This is the standard (unmodified, or non-ARCA) cap analog (Life Technologies, Cat. No. AM8048).
2. $m_2^{7,3'\text{-}O}$GpppG (3′-ARCA) (Life Technologies, Cat. No. AM8045). This is the original ARCA (9).
3. $m_2^{7,2'\text{-}O}$GpppG (2′-ARCA). Its translational properties are indistinguishable from the 3′-ARCA (10).
4. $m_2^{7,2'\text{-}O}$Gpp$_S$pG, D1 (β-S-ARCA D1), a diastereomer of $m_2^{7,2'\text{-}O}$Gpp$_S$pG (β-S-ARCA) (26]. When incorporated into mRNA, it gives enhanced expression of protein in immature mouse dendritic cells (25) and whole animals (24).
5. $m_2^{7,2'\text{-}O}$Gpp$_S$pG, D2 (β-S-ARCA D2) (26), another diastereomer of β-S-ARCA. mRNA containing this analog has higher translational efficiency and stability than the corresponding mRNA containing 2′-ARCA or β-S-ARCA D1 in mouse mammary epithelial cells (21).
6. $m_2^{7,2'\text{-}O}$Gpp$_{BH3}$pG (β-B-ARCA D2), a diastereomer of $m_2^{7,2'\text{-}O}$Gpp$_{BH3}$pG (23). This analog is similar to β-S-ARCA D2 except the S has been replaced with BH$_3$. When incorporated into mRNA, it confers the highest in vitro translational efficiency of all analogs described here and is one of three (along with BTH and $m_2^{7,2'\text{-}O}$GppNHpG) that confer the highest stability to mRNA in HeLa cells (23).
7. m^7Gpp$_{BH3}$pm^7G (BTH) (23). This analog does not have modifications on either the 2′ or 3′ positions of m^7Guo but nonetheless serves as an ARCA because both nucleoside moieties contain m^7G. We abbreviate it "BTH" for *B*orano *T*wo *H*eaded. When incorporated into mRNA, it confers the highest stability in HeLa cells (23).

2.2. Construction of a Plasmid Template for Synthesis of Luc-A$_{50}$ mRNA

1. p*luc*-A+ (10), which contains the entire firefly luciferase mRNA sequence downstream of the T7 promoter in pGEM4® (Promega, Cat. No. P2161) plus a 3′-terminal 31-nt poly(A) tract.
2. 100 μM of the following HPLC-purified oligonucleotides: 5′-C(A)$_{49}$AAACATTGCGTT-3′ and 5′-AACGCAATGTTT-(T)$_{49}$GAGCT-3′ (see Note 1).

3. Restriction enzymes BsrDI, HpaI, and SacI (New England Biolabs, Cat. No. R0574, R0105, and R0156, respectively).
4. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.
5. 3 M sodium acetate, pH 5.2.
6. 100% and 70% ethanol.
7. SpeedVac concentrator.
8. Horizontal electrophoresis system.
9. Agarose.

2.3. In Vitro Synthesis of Luc-A_{50} mRNA with Different Caps

1. DEPC-treated water.
2. 5× Transcription buffer: 200 mM Tris–HCl, pH 7.9, 30 mM $MgCl_2$, 10 mM spermidine (see Note 2).
3. 100 mM dithiothreitol (DTT).
4. 10 mg/mL bovine serum albumin (BSA).
5. RNase Inhibitor, 20 units/μL (Applied Biosystems, Cat. No. N8080119).
6. ATP, 10 mM.
7. UTP, 10 mM.
8. CTP, 10 mM.
9. GTP, 10 mM.
10. Cap dinucleotide, 10 mM (see Subheading 2.1).
11. DNA template, linearized, 1 μg/μL.
12. T7 RNA polymerase, 10 units/μL.
13. RNase-free DNase RQ1, 1 unit/μL (Promega, Cat. No. M6101).
14. E.Z.N.A. Total RNA Miniprep Kit I consisting of HiBind RNA spin columns, collection tubes, RNA Wash Buffer I, and RNA Wash Buffer II (Omega Bio-Tek, Cat. No. R6834-01).
15. 100% ethanol.
16. 70% ethanol.
17. Horizontal electrophoresis system.
18. Agarose.
19. Formaldehyde.

2.4. Introduction of Synthetic mRNAs into Mammalian Cells by Nucleoporation

1. Cell culture medium: DMEM high glucose containing 10% fetal bovine serum and penicillin/streptomycin antibiotics.
2. Nucleofector™ II nucleoporator (Lonza, Cat. No. AAD-1001).
3. Nucleofector™ solution R (Lonza, Cat. No. AAF-1001B).
4. Phosphate-buffered saline (PBS): Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in distilled

water. Adjust pH to 7.4 with HCl. Make up to 1,000 mL. Sterilize by autoclaving.

5. 1.5-mL Eppendorf tubes and cell culture dishes.

2.5. Analysis of Translational Efficiency in Cells

1. Cell Culture Lysis Reagent, 5× (Promega, Cat. No. E153A). Dilute to 1× before use.
2. Luciferase Assay System (Promega, Cat. No. E1500).

2.6. Analysis of mRNA Stability in Cells

1. E.Z.N.A. Total RNA Kit 1 (Omega Bio-Tek, Cat. No. R6834-01).
2. PBS (see Subheading 2.4).
3. 70% ethanol.
4. 10× RQ1 DNase buffer (Promega, Cat. No. M6101).
5. RNase-free DNase RQ1, 1 unit/μL (Promega, Cat. No. M6101).
6. 10× Reverse transcription buffer: 100 mM Tris–HCl, pH 8.3, 500 mM KCl.
7. 25 mM $MgCl_2$.
8. 10 mM dNTP mixture (2.5 mM for each of the four dNTPs).
9. 50 μM Random Hexamers (Cat. No. N8080127) (Applied Biosystems).
10. RNase Inhibitor, 20 units/μL (Applied Biosystems, Cat. No. N8080119).
11. MultiScribe™ Reverse Transcriptase, 50 units/μL (Applied Biosystems, Cat. No. 4311235).
12. RNase-free water (see Note 3).
13. PerfeCTa SYBR Green SuperMix for iQ (Quanta, Cat. No. 95053-100).
14. Mixture of primers to amplify firefly luciferase cDNA: forward, 5′-GGATGGAACCGCTGGAGAG-3′; reverse, 5′-GCATACGACGATTCTGTGATTTG-3′, 10 μM each.
15. Mixture of primers to amplify human GAPDH cDNA: forward, 5′-CGAGCCGCCTGGATACC-3′; reverse, 5′-CAGTTCCGAAAACCAACAAATAGA-3′, 10 μM each.
16. iCycler IQ real-time PCR detection system (Bio-Rad, Cat. No. 170-8740).
17. PC computer with KaleidaGraph software (Synergy Software, version 3.06).

2.7. Production of Proteins in Experimental Animals

1. mRNA (see Subheading 2.3).
2. RNase-free water and RNase-free 10× PBS (Life Technologies, Cat. No. AM9625) for mRNA dilution to achieve a final composition of 1× PBS (see Note 4).
3. Sterile and RNase-free syringes and needles.

4. *D*-luciferin.
5. Bradford assay kit (Bio-Rad, Cat. No. 500-0001).
6. Spectrophotometer.
7. Bioluminescence imaging system.
8. Isoflurane.

3. Methods

3.1. Construction of a Plasmid Template for Synthesis of Luc-A_{50} mRNA

1. Anneal oligonucleotides by mixing equal volumes from the stocks, heating 100°C for 10 min, and cooling slowly to room temperature for few hours or overnight.
2. Ligate the resulting double stranded DNA fragment into the SacI/HpaI site of p*luc*-A+ by using the Rapid DNA Ligation Kit, generating p*luc*-A50.
3. Digest 10 μg of p*luc*-A50 in a 100-μL reaction with 20 units of BsrDI to obtain a linear template for transcription reaction.
4. Incubate the reaction mixture for 2 h at 65°C.
5. Add 100 μL of TE buffer.
6. Extract the reaction mixture once with an equal volume (200 μL) of phenol and once with an equal volume (200 μL) of chloroform.
7. Transfer the aqueous phase to a new tube, add 20 μL of sodium acetate, and mix well.
8. Add 2.5 volumes (500 μL) of ice-cold 100% ethanol and store the tube at –20°C for 1 h.
9. Recover the precipitated DNA by centrifugation at 12,000 × *g* for 20 min at 4°C.
10. Remove the supernatant by aspiration.
11. Add 1 mL of ice-cold 70% ethanol, vortex briefly, and centrifuge at 7,500 × *g* for 5 min at 4°C.
12. Remove the supernatant and dry the DNA pellet in a SpeedVac concentrator.
13. Resuspend the DNA in 40 μL of water.
14. Check DNA concentration by UV absorbance at 260 nm and verify integrity of DNA by electrophoresis on a 1% agarose gel (see Note 5).

3.2. In Vitro Synthesis of Luc-A_{50} mRNA with Different Caps

1. Prepare 200 μL of transcription reaction mixture in a 1.5-mL centrifuge tube by adding the following reagents at room temperature in the order listed: 40 μL 5× transcription buffer, 20 μL DTT, 2 μL BSA, 5 μL RNase Inhibitor, 10 μL ATP,

10 µL UTP, 10 µL CTP, 2 µL GTP, 20 µL cap analog, 3 µL DNA template, and 10 µL of RNA polymerase, making up the volume with DEPC-treated water.

2. Incubate 1 h at 37°C.
3. To increase the yield of RNA, add an additional 10 µL of RNA polymerase and incubate for another 1 h at 37°C.
4. Treat the reaction mixture with 10 units of DNase for 30 min at 37°C to remove the DNA template.
5. Purify RNA using an E.Z.N.A. Total RNA Miniprep Kit I as follows (see Note 6).
6. Add 500 µL of TRK Lysis Buffer to the reaction and mix thoroughly by vortexing or pipetting up and down a few times.
7. Add an equal volume (700 µL) of 70% ethanol and mix thoroughly by pipetting up and down a few times.
8. Apply the sample to the column inserted into a 2-mL collection tube.
9. Centrifuge at 10,000 × *g* for 1 min at room temperature and discard the flow-through.
10. Add 500 µL of RNA Wash Buffer I and repeat centrifugation.
11. Add 500 µL of RNA Wash Buffer II and repeat centrifugation (see Note 7).
12. Repeat this step one more time.
13. Remove the flow-through and centrifuge the column for 2 min to completely dry the matrix.
14. Transfer the column into a new 1.5-mL centrifuge tube and elute the RNA by adding 50 µL of DEPC-treated water (see Note 8).
15. Centrifuge for 1 min at 10,000 × *g*.
16. Check RNA concentration by absorbance at 260 nm (see Note 9) and verify RNA integrity by electrophoresis on a 1.2% agarose gel containing formaldehyde.

3.3. Introduction of Synthetic mRNAs into Mammalian Cells by Nucleoporation

1. Seed HeLa cells at 30–40% confluency 1 day prior to nucleoporation. For a 150-mm dish, seed 1.5 to 2×10^6 cells.
2. Pre-warm the supplemented Nucleofector™ Solution R to room temperature. Pre-warm an aliquot of culture medium at 37°C in a 50-mL tube.
3. Harvest the cells when they have reached ~70% confluency as follows. Remove the medium from the cell culture dish. Wash cells once with PBS. Add 1.5 mL of 0.05% trypsin and 2 mM EDTA in PBS and incubate cells 5 min at 37°C in an incubator with 5% CO_2. Add 5 mL of fresh medium and collect cells by centrifugation 800 × *g* for 5 min. Count the cells and incubate them in fresh media at 37°C for 30 min.

4. Pellet the cells by centrifugation and remove the medium. Wash with PBS once and pellet by centrifugation again.
5. Resuspend the cells in 100 μL of Nucleofector™ Solution R. Use 5×10^5 to 5×10^6 cells per nucleoporation. Using fewer cells leads to a major increase in cell mortality; using more cells causes inefficient delivery of RNA.
6. Add 1 μg mRNA to the cells in nucleoporation solution and mix well.
7. Transfer the mixture containing cells and RNA to the nucleoporator cuvette and carry out nucleoporation with a Nucleofector™ II, using program I-13 (see Note 10).
8. Add 500 μL pre-warmed medium to the cuvette and transfer contents to a 15-mL tube containing 5 mL of pre-warmed medium. Spin down the cells and remove the medium.
9. Resuspend the cells in fresh warm medium and withdraw 0.5×10^6 cells for each time point, e.g., every 15 min. For time points up to 1 h, keep cells in 1.5-mL Eppendorf tubes shaking at 37°C in a water bath. For time points greater than 1 h, seed each aliquot of 0.5×10^6 cells onto a 35-mm dish and place in a 37°C incubator with 5% CO_2. For each time point, wash cells with PBS, and add lysis buffer (see Note 11).

3.4. Analysis of Translational Efficiency in Cells

1. Shake aliquots of 0.5×10^6 cells in Eppendorf tubes in a 37°C water bath for various times after nucleoporation up to 60 min, e.g., 12, 24, 36, 48, and 60 min.
2. Spin down cells at 800 × *g* for 1 min, aspirate off the medium, and wash cells with PBS.
3. Spin down cells to remove PBS, resuspend the pellet in 200 μL 1× Cell Culture Lysis Reagent, vortex for 10 s, and place on ice for 10 min.
4. Spin down the cell debris at 4°C at 9,000 × *g* for 2–3 min.
5. Transfer the supernatant to a new Eppendorf tube and freeze at −80°C until use.
6. Determine the protein concentration in the lysate using the Bradford assay.
7. Add 5–10 μL of protein extract into the luciferase assay reagent from a Luciferase Assay System kit (Promega).
8. Transfer the sample to a luminometer and determine luciferase activity (see Note 12).
9. Normalize luciferase activity by protein concentration of each sample.
10. To compare translational efficiencies between different nucleoporated groups, normalize luciferase activity to the amount of luciferase mRNA present in the cells at 0 min (see Subheading 3.5 and Note 11).

3.5. Analysis of mRNA Stability in Cells

3.5.1. mRNA Isolation from HeLa Cells (see Note 13)

1. Take cells incubated in 1.5-mL tubes from step 9 of Subheading 3.3, spin in an Eppendorf centrifuge at 800 × *g* for 2 min, wash the pellet with 1 mL PBS, centrifuge, and discard the PBS. Take cells grown in monolayer from step 9 of Subheading 3.3, carefully aspirate the cell culture medium, wash the cells with 1 mL of PBS, and remove the PBS by aspiration.

   Add 400 μL TRK lysis buffer from the E.Z.N.A. Total RNA Kit 1 to the cells pelleted in Eppendorf tubes, vortex the tube, place on ice for 5 min, and freeze at −80°C. Add 400 μL of the same buffer to the cells grown in monolayer, place the dish on ice for 5 min, transfer the lysate to a new Eppendorf tube, and freeze at -80°C (see Note 14).
2. Thaw lysates by incubating tubes in room temperature water for 2 min.
3. Add 400 μL of 70% ethanol and mix thoroughly by vortexing.
4. Apply the samples to HiBind RNA spin columns placed in 2-mL collection tubes (see Note 15).
5. Centrifuge at 13,000 × *g* for 30 s in an Eppendorf centrifuge, discard the flow-through, and wash the spin columns with 350 μL of RNA Wash Buffer I.
6. Centrifuge at 13,000 × *g* for 30 s and discard the flow-through.
7. For each HiBind RNA column, prepare a DNase solution by combining 5 μL of 10× RQ1 DNase buffer, 42 μL of RNase-free water, and 3 μL of RNase-free DNase RQ1.
8. Pipet the DNase solution directly onto the HiBind RNA matrix of each column and incubate at room temperature for 30 min.
9. Add 400 μL of RNA Wash Buffer I and leave the columns at room temperature for 2 min.
10. Centrifuge at 13,000 × *g* for 30 s, discarding the flow-through.
11. Wash the columns with 500 μL of RNA Wash Buffer II, and centrifuge at 13,000 × *g* for 30 s, discarding the flow-through.
12. Wash the columns with another 350 μL of RNA Wash Buffer II and centrifuge at 13,000 × *g* for 30 s, discarding the flow-through.
13. Centrifuge the HiBind spin RNA columns one more time at 16,400 × *g* for 2 min to completely dry the matrix.
14. Place the spin columns into new 1.5-mL centrifuge tubes and elute the RNA with 40 μL of RNase-free water by centrifugation at 13,000 × *g* for 1 min.

15. Determine the concentration and purity of RNA by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer (see Note 16).

3.5.2. Quantitative Reverse Transcription/Real-Time PCR (qRT-PCR) (see Note 17)

1. Prepare a Master Mix by adding the reagents in the order and proportion as follows: 2 μL of 10× Reverse Transcription Buffer, 3.7 μL of RNase-free water, 4.4 μL of 25 mM $MgCl_2$, 4 μL of 10 mM dNTP mix, 1 μL of 50 μM Random Hexamers, 0.4 μL of RNase Inhibitor, and 0.5 μL of MuLV Reverse Transcriptase (see Note 18).
2. Add 4 μL of RNA sample containing 400 ng of total RNA to the Master Mix, the final volume being 20 μL (see Note 19).
3. Carry out reverse transcription in a thermocycler with the program 25°C for 10 min, 48°C for 30 min, 95°C for 5 min, and hold at 4°C.
4. Prepare one Master Mix for PCR amplification of firefly luciferase cDNA and a second Master Mix for PCR amplification of GAPDH cDNA, each containing 12.5 μL of PerfeCTa SYBR Green SuperMix for iQ, 6 μL of water (see Note 18), and 1.5 μL of a 10 μM mixture of forward and reverse primers for either firefly luciferase or GAPDH cDNA.
5. Dilute cDNA samples twofold with water and pipette 5 μL of each sample into a 96-well PCR plate in duplicate.
6. Add 20 μL of the Master Mix for amplification of either firefly luciferase or GAPDH cDNA.
7. Detect amplification with the iCycler IQ real-time PCR detection system by using following program: 3 min at 95°C for polymerase activation; 40 cycles of 15 s at 95°C and 1 min at 60°C.
8. Calculate luciferase mRNA levels using the absolute standard curve method as described in User Bulletin No. 2 for ABI Prism 7700 Sequence Detection System.

3.5.3. Determination of mRNA Half-Life ($t_{1/2}$)

1. Express the amount of luciferase mRNA remaining at different time points after nucleoporation as a percentage of mRNA at time zero.
2. Fit the plot of the percentage of remaining mRNA versus time to a single-exponential function by using the plotting and curve-fitting software KaleidaGraph.
3. From the observed first-order rate constant (k) obtained from the fit for decay of each mRNA, determine $t_{1/2} = \ln 2/k$.
4. When a lag in the decay of mRNA is observed, fit only the data points occurring after the lag to a single-exponential function.

3.6. Production of Proteins in Experimental Animals

1. Dilute RNA (10–30 μg) coding for firefly luciferase in 1× PBS (see Note 4) and inject the RNA into anesthetized mice (see Note 20). The RNA can be injected subcutaneously, intradermally, or intranodally (see Note 21).
2. At the time of bioluminescence imaging (usually 18 h after RNA injection if only one measurement is to be performed), start by preparing an aqueous solution of luciferin (150 mg/kg body weight per mouse).
3. Anesthetize the mice by isoflurane inhalation (see Note 20).
4. Inject luciferin solution intraperitoneally and wait for at least 5 min before imaging.
5. Perform in vivo imaging by recording the luciferase signal emitted from the injection site (see Note 22). Figure 5 shows an example of luciferase expression from RNAs capped with either ARCA or β-S-ARCA D1, injected intranodally, demonstrating that mRNA containing the latter cap is more effective in stimulating protein production in whole animals.

4. Notes

1. The annealed double-stranded oligonucleotides contain SacI and HpaI restriction sites as well as a BsrDI site just upstream of the HpaI site. When the plasmid is linearized with BsrDI and transcribed, the RNA generated will contain a 3′-terminal poly(A) tract with no heterologous (non-A) nucleoside residues.
2. Store this and all the other buffers at −20°C.
3. Use DEPC-treated water to prepare solutions of PBS, 10× Reverse transcription buffer, KCl, $MgCl_2$, and dNTPs.
4. In our experience, RNA diluted into an isotonic buffer gives higher protein expression than RNA diluted into water. Optimal buffers should be identified for each application.
5. A typical DNA yield at this point is 5–7 μg. Integrity of the DNA on agarose gels is verified if there is only one band, provided the plasmid contains only BsrDI site. Also, the original plasmid and digested plasmid should show different bands on agarose electrophoresis.
6. The procedure described is given in more detail in the manufacturer’s instructions. Alternatively, RNA may be purified with one of the following methods: phenol/chloroform extraction followed by LiCl precipitation, Trizol Reagent (Invitrogen), or columns for RNA purification from companies other than Omega Bio-Tek.

7. RNA Wash Buffer II must be diluted with 100% ethanol before use.
8. Add the water directly into the center of the column matrix.
9. The concentration of the RNA should be ~500 ng/μL.
10. Electroporation can also be used to deliver synthetic mRNAs into cells, but it requires more reagents and cells since it causes greater cell death.
11. Time points to follow RNA decay can be taken every 15 min until 1 h and then every 30 min or 1 h afterwards. For luciferase production, samples can be collected every 12 min up to 60 min.
12. Measure luciferase activity in duplicate or triplicate, and include a blank in the same experiment (5 μL lysis buffer added into the luciferase assay reagent).
13. Carry out all procedures at room temperature unless otherwise specified.
14. Do not keep lysates on ice until the last sample is collected. Usually, collection of samples takes 2–8 h. During that time a significant amount of RNA can be degraded if the lysates are left on ice.
15. HiBind RNA spin columns, collection tubes, RNA Wash Buffer I and RNA Wash Buffer II are supplied in the E.Z.N.A. Total RNA Kit 1.
16. For spectrophotometric analysis, dilute the RNA samples in 10 mM Tris–HCl, pH 8.0. DEPC-treated water is slightly acidic and can lower absorbance values. Typical yields of total RNA from 5×10^5 HeLa cells are 6–8 μg. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA. A ratio of ~2.0 is generally accepted as indicating "pure" RNA. If the ratio is appreciably lower, it may indicate the presence of protein or other contaminants that absorb at 280 nm; if appreciably higher, it may indicate the presence of phenol.
17. During this step, it is important to prevent contamination of reagents. Treat your working area with RNaseZap (Ambion) to remove RNase contamination. Use pipette tips with filters. Do not work in an area where experiments involving DNA are conducted.
18. For the Master Mix, it is convenient to make enough reagent for all samples. This includes two extra reaction volumes to accommodate reagent losses during pipetting.
19. Pipet each reaction mixture in a 0.2-mL PCR tube with attached dome cap.
20. Mice can be anesthetized either by inhalation (e.g., isoflurane) or by intraperitoneal injection of Ketamine (120 mg/kg body weight) and Xylazine (2 mg/kg body weight).

21. RNA should be injected using sterile RNAse-free syringes. For intranodal RNA injection, it is necessary to surgically free the lymph node of choice before injection.
22. Different instrumentation necessitates different protocols for data acquisition. Therefore no detailed protocol can be given here.

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Chapter 5

Nonviral, Cationic Lipid-Mediated Delivery of mRNA

James G. Hecker

Abstract

Appropriate gene delivery systems are essential for successful gene therapy in clinical medicine. Cationic lipid-mediated delivery is an alternative to viral vector-mediated gene delivery where transient gene expression is desirable. However, cationic lipid-mediated delivery of DNA to post-mitotic cells is often of low efficiency, due to the difficulty of DNA translocation to the nucleus. Rapid lipid-mediated delivery of RNA is preferable to nonviral DNA delivery in some clinical applications, because transit across the nuclear membrane is not necessary. Here we describe techniques for cationic lipid-mediated delivery of RNA encoding reporter genes in a variety of in vitro cell lines and in vivo. We describe optimized formulations and transfection procedures that we have previously assessed by flow cytometry. RNA transfection demonstrates increased efficiency relative to DNA transfection in nondividing cells. Delivery of mRNA results in onset of expression within 1 h after transfection and a peak in expression 5–7 h after transfection. These results are consistent with our in vivo delivery results, techniques for which are shown as well. Longer duration and the higher mean levels of expression per cell that are ultimately obtained following DNA delivery confirm a continuing role for DNA gene delivery in clinical applications that require long term transient gene expression. RNA delivery is suitable for short-term transient gene expression due to its rapid onset, short duration of expression, and greater efficiency, particularly in nondividing cells.

Key words: Nonviral, Lipid-mediated, Gene delivery, Transfection, RNA, DNA, siRNA, Primary neurons, Post-mitotic, Molecular therapy, Transient, CHO, NIH3T3

1. Introduction

Gene therapy has the potential to significantly advance clinical medicine. But the risks and duration of gene delivery should be closely matched to the proposed clinical application (1). Long term expression after gene therapy is useful for diseases which require chronic levels of protein expression, such as inherited enzyme deficiencies, or cancer, and for these diseases viral vectors may offer advantages. For clinical applications in which only short-term gene

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expression is required or warranted, the delivery of nucleic acids by means of nonviral lipids provides a more favorable risk/benefit ratio. Lipid-mediated transfection also offers other advantages over viral vectors, most notably safety, low immunogenicity, ease of preparation, and the ability to transfect macromolecules of nearly unlimited size (2, 3).

Cationic lipid-mediated gene transfer is particularly suited for transient gene expression, both in basic research and in selected clinical applications. Cationic lipids are commonly comprised of a polar head group and nonpolar symmetric or dissymmetric carbon based tail (see Note 1). Negatively charged nucleic acids condense and self-assemble into heterogeneous complexes of lipids and nucleic acids when mixed with cationic lipids (4). The structure and size of these complexes affect transfection efficacy and vary with temperature, concentration, charge ratio, buffer, time, and lipid composition. These lipid–nucleic acid complexes protect nucleic acids from degradation in the extracellular environment (5). Numerous laboratories (6, 7), including our own (8), have investigated the limiting parameters of cationic lipid-mediated transfection with the goal of improving transfection efficiency (9).

For DNA transfection there are four general barriers: (1) transport of the nucleic acid/lipid complex in the extracellular environment; (2) association and uptake of the nucleic acid/lipid complex by the target cell (10); (3) intracellular DNA release from the nucleic acid/lipid complex (11); and (4) translocation of DNA to the nucleus (12). The primary barrier to DNA transfections in post mitotic cells is assumed to be DNA translocation to the nucleus (13). While cationic lipid-mediated transfections work well with many types of cells (2, 3, 6), transfection of primary cells often remains a problem (9, 14), and this transfection difficulty is generally attributed to markedly reduced or absent mitotic activity in these almost exclusively post-mitotic cells (14, 15). In proliferating cells, nuclear translocation is mainly passive, occurring during mitosis as the nuclear membrane breaks down (10, 16, 17). Some nuclear translocation does still occur in non-proliferating cells, probably the result of passive movement through the nuclear pore complex (NPC) (18, 19).

Using RNA instead of DNA eliminates the necessity for nuclear translocation and thus has the potential to greatly improve transfection of post-mitotic cells. Lipid-mediated RNA and DNA delivery to proliferating cells (20) as well as intramuscular injection of naked RNA and DNA have been previously described (21). Methods for avoiding the necessity of nuclear translocation of DNA have also been reported, such as delivery of T7 promoter DNA plasmid systems to T7 polymerase expressing cells (22), but have limited clinical applications.

We have compared numerous lipids, cationic and otherwise, in a wide variety of in vitro and in vivo applications, including in vivo imaging, primary neuronal cells, and proliferation inhibited dividing cells (15). Our studies show that RNA is two to five times more efficient at cell transfection than DNA based on the percentage of cells transfected, is rapidly and robustly expressed, and offers safety advantages over viral vectors (15, 23). DNA is used as a control for mRNA transfections. DNA is more stable, and gives a higher level of expression under most cases, although with a longer time to expression, and a longer duration of expression. *In trouble shooting it is advisable to have DNA transfection controls in parallel.*

We recommend the commercially available lipid TransFast™ Transfection Reagent (Promega, Madison, WI). It is in our long experience the closest in efficiency and low cytotoxicity to the novel lipid MLRI, which is available by special order but not commercially. see Note 1. TransFast™ Transfection Reagent is comprised of the synthetic cationic lipid (+)-N,N{bis (2-hydroxyehyl)-N-methyl-N-{2,3-di(tetradecanolyloxy)propyl} ammonium iodide and the neutral lipid, DOPE (Promega, WI). Liposome reagents specifically designed for transfection applications often incorporate synthetic cationic lipids, such as the neutral lipid DOPE or cholesterol, to improve efficacy. DOPE has been demonstrated to enhance the gene transfer ability of certain cationic lipids (24). Transfast is supplied by Promega as a lipid film in glass vials under argon gas that must be rehydrated with DDW (nuclease and RNase free). We have optimized transfection formulations of GFP encoding RNA and DNA vectors using flow cytometry, by varying charge ratio, formulation time, concentration, and temperature, to obtain the maximum percentage of GFP expressing cells. *Ideally each cell line of interest should have optimal formulations confirmed before proceeding to* in vivo *experiments.*

This protocol is focused on mRNA delivery into primary neurons. However, it is quite practical at first to work out the main parameters of liposomal formulation on fast growing, easily available cell types. That is why we include the experiments on CHO cells The protocol includes the use of GFP and luciferase reporter transcripts. Both can serve as suitable reporter constructs and controls. Although the measurement of GFP expression by flow cytometry can be preferable for cell line transfection, flow analysis of neuronal cells is more difficult, and luciferase reporter is more suitable for analysis of neuronal transfection.

2. Materials

2.1. Cells

1. CHO cells (ATCC, Rockville, MD).
2. Primary neuronal cells dissected from the cortex of day 17 Sprague–Dawley rat fetuses.

2.2. Plasmids and Transcripts

1. β-Globin luciferase plasmid, which contains β-globin stabilizing elements, was used for time course experiments in primary neuronal cells (gift of Jon Wolf, MD, University of Wisconsin) (20, 21). β-globin is one of the most stable naturally occurring mRNAs found in nature, and the stabilizing elements from β-globin mRNA have been used in in vitro mRNA transcripts to design long lived mRNA. We did not see significant differences in stability between the various mRNAs we produced and analyzed. To produce the β-globin stabilized transcripts, cloning procedures were carried out essentially as described in Molecular Cloning: A Laboratory Manual (25). Xenopus laevis β-globin sequences were derived from the plasmid pSP64 T (26), with the 5′ β-globin sequences obtained as the HindIII/Bgl II fragment and the 3′ β-globin sequences released as the Bgl II/EcoRI fragment. These 3′ sequences include a terminal polynucleotide tract of A23C30. T7 RNA polymerase transcription templates, as well as various mRNAs produced from them, are outlined in ref (20).

2.3. Equipment

1. 5% CO_2 incubator at 37°C.
2. Low speed centrifuge.
3. Dual channel FACScan or multichannel Caliber flow cytometer (Becton Dickinson, San Jose, CA) equipped with a single 488 nm argon laser (GFP fluorescence measured with a 530 nm band pass filter and Tri-Color fluorescence measured with a 675 band pass filter).
4. CellQuest (Becton Dickinson) or equivalent flow cytometry analysis software.
5. Monolight 2010 (Analytical Luminescence Laboratory, now Becton-Dickinson) or equivalent.
6. Stereotaxic small animal surgery frame (Stoelting, Wood Dale, IL).
7. Syringe infusion pump (Model 101, Stoelting).

2.4. Supplies

1. 75 cm^2 cell culture flasks.
2. 60 mm Petri dishes.
3. 15 ml sterile tube.
4. Pasteur pipette tubes.
5. 40 μm nylon nets.
6. Intraventricular canula.
7. Microscope slides (Columbia Diagnostics, Inc., Springfield, VA) poly-l-lysine-coated and air-dried for a minimum of 2 h.

2.5. Media

1. HAMS F12 media (Life Technologies, Gaithersburg, MD).
2. DMEM media.
3. OptiMem media.
4. 10% bovine calf serum.
5. PBS.
6. Ice cold sterile PBS.
7. Neural Basal Medium (NBM), containing 0.5 mM l-glutamine, 1× B27, 50 ng/ml neural growth factor, supplemented with 0.25% trypsin-EDTA and 20 μl DNase I.

2.6. Templates for RNA Synthesis

1. mRNA of your desired sequences using standard techniques (in vitro transcription and purification using RNAse-free laboratory techniques, as in Red Book or other standard lab reference) (see Note 2).
2. eGFP and luciferase mRNA transcripts as controls for optimization (β-globin eGFP is shown in Methods) (see Note 3).
3. Phenol–chloroform for mRNA purification.

2.7. Nucleic Acid Delivery

1. Restriction enzymes (we used Nco1, Nde1, Dra I and Xba1).
2. Proteinase K.
3. Phenol–chloroform.
4. Ethanol.
5. T7 mMessage mMachine transcription kit (Ambion, Austin, TX).
6. Enhanced Luciferase assay Kit (BD Bioscience).
7. TransFast™ Transfection Reagent (Promega, Madison, WI).
8. Neutral lipid DOPE (Promega, WI) or cholesterol.
9. eGFP or luciferase pDNA control vectors (Clontech).
10. Trypsin.
11. Dulbecco's phosphate buffered saline with Ca^{2+} (DPBS).
12. Annexin V conjugated with biotin (CalTag, Burlingame, CA) in DPBS.
13. Streptavidin conjugated with a Tri-Color fluorophore (CalTag, Burlingame, CA) in DPBS.
14. Lysis buffer.
15. CSF (cerebral spinal fluid).

2.8. Tissue Preparation for Reporter Protein Localization After In Vivo mRNA Delivery

1. Iced saline for perfusion.
2. Iced 4% paraformaldehyde in 0.1 *M*, pH 7.4 sodium phosphate-buffered saline (PBS).
3. Paraformaldehyde fixative at 4°C.
4. PBS containing 20% glycerol at 4°C.

2.9. Diaminobenzidine mmunohistochemistry

1. Diaminobenzidine (DAB).
2. 24-well plates for free-floating sections.
3. ExtrAvidin® peroxidase system (Sigma, St. Louis, MO).
4. Poly-l-lysine-coated microscope slides for slide-mounted sections (Columbia Diagnostics, Inc., Springfield, VA).
5. H_2O_2.
6. 4% paraformaldehyde (described above) 0.1% H_2O_2.
7. Blocking buffer 0.3% Triton X-100, 3% bovine serum albumin (BSA), 10% normal goat serum (NGS), Ca^{+2} and Mg^{+2}-free PBS.
8. Biotin-conjugated secondary Ab (biotinylated goat anti-mouse BA-9200, Vector Labs) tertiary horseradish peroxidase-conjugated probe (ExtrAvidin® peroxidase system).
9. 50 m*M* Tris–HCl at pH 7.6.
10. 0.5 mg/ml 3,39-DAB with 0.03% H_2O_2 as the peroxidase substrate.
11. Mouse monoclonal neuron specific nuclear protein anti-NeuN (MAB377, 1:50; Chemicon).
12. Rabbit polyclonal antiluciferase antibody (CR2029R, 1:50; Cortex Biochem, CA).

2.10. Documentation of Results

1. Nikon 600 microscope with camera mount.
2. Photoshop and Polaroid SprintScan slide scanner for film negatives or slides.
3. Fuji Pictrography 3000 (Fuji Photo Film, Elmsford, NY), or
4. Nikon Eclipse TS100 Inverted microscope or equivalent.
5. High resolution digital camera (SPOT, Diagnostic Instruments) or equivalent.

3. Methods

3.1. CHO Cells

1. Culture CHO cells in 75 cm^2 cell culture flasks with HAMS F12 media at 37°C in a 5% CO_2 environment (see Note 4).
2. Split cells 48 h prior to transfection.
3. Plate at 60% confluence.

3.2. Primary Neuronal Cells

1. Remove fetal rat brains and place in ice cold sterile PBS (see Note 5).
2. Dissect cortex and clear meninges.

3. Transfer cortical sections to a 60 mm Petri dish containing 4 ml PBS.
4. Mince into pieces of approximately 1 cubic millimeter in size.
5. Transfer into a 15 ml sterile tube.
6. Add 4 ml Neural Basal Medium and shake for 30 min at 37°C.
7. Extrude the resultant suspension through a Pasteur pipette tube to eliminate residual clumps of brain tissue.
8. Filter through 40 μm nylon net to yield single cell suspensions.
9. Spin cells and resuspend in Neural Basal Medium.
10. Seed 1×10^6 cells per ml onto 24-well tissue culture dishes which have been pre-coated with poly-l-lysine.
11. Culture cells in 5% CO_2 incubator at 37°C.
12. Maintain in culture for 2 weeks prior to transfection to allow the development of the phenotype of mature human cortical neurons.
13. Replace one half of the medium every 3–4 days with medium containing fresh neural growth factor (see Note 6).

3.3. β-Globin Vector for mRNA Transcript (see Note 7)

1. Subclone eGFP into β-globin vector using Nco1 and Xba1 restriction enzymes to form β-globin enhanced Green Fluorescent Protein (β-globin eGFP) vector.
2. Linearize β-globin eGFP with restriction enzyme Dra I after elimination of RNases by proteinase K treatment and phenol–chloroform extraction.
3. Precipitate in ethanol.
4. Transcribe linearized DNA templates in vitro using T7 mMessage mMachine transcription kit.

3.4. Formulation of mRNA with Lipid to Form Lipoplexes and CHO Cell Transfections (see Note 8)

Use both GFP and Luc transcripts and plasmid DNAs for optimization of lipoplex formulations

1. Day One, plate cells.
2. Calculate and prepare enough mRNA (or DNA as control) and Transfast for as many wells as your experiment calls for. For in vitro experiments in 24-well plates 1 μg of nucleic acid (mRNA or plasmid DNA) is added to OptiMem for each well of the 24-well plate (see Note 9).
3. Rehydrate Transfast lipid in nuclease and RNAse-free DDW water and store at −4°C overnight.
4. Day 2, dilute mRNA in 20 μl OptiMem solution.
5. Thaw Transfast.

6. Add nuclease- and RNAse-free nucleic acids of sequence of interest to OptiMem for a final volume of 200 μl per well of 24-well plate.
7. Vortex.
8. Add lipid to produce a 3:1 lipid:nucleic acid charge ratio in 200 μl per well of 24-well plate (see Note 9).
9. Add thawed Transfast to NA (mRNA or DNA as control) and vortex 15 s.
10. Incubate for 45–60 min at 37°C (see Note 10).
11. Aspirate off growth medium from CHO cells.
12. Transfect cells in each well of 24-well plates with 1 μg of mRNA (or DNA as control) (see Note 9).
13. Add 200 ml of transfection solution to each well of a 24-well plate (see Note 11).
14. Incubate in 37°C CO_2 incubator for 60 min.
15. Add growth medium to cells but do not aspirate off transfection solution.
16. Return to incubator for appropriate time (5–8 h for mRNA, 24–48 h for DNA).
17. Perform appropriate assay, i.e., flow cytometry or Luminometer for GFP or luciferase (see Note 12).
18. The final solution, in 200 μl, is vortexed and incubated for 1 h at room temperature (RT) prior to aspiration of growth media from the cells and application of transfection formulation to cells.
19. After 1 h of incubation supplement cells with 1 ml of growth media.
20. Harvest cells for flow cytometry analysis 7 h after RNA transfections (24 h after GFP DNA control transfections).

3.5. Cell Toxicity Analysis (Biotin-Conjugated Annexin V)

1. Trypsinize CHO cells and wash twice in Dulbecco's phosphate buffered saline with Ca^{2+} (DPBS).
2. Resuspend and incubate 30 min in annexin V conjugated with biotin in DPBS.
3. Wash again with DPBS and resuspend cells 30 min in streptavidin conjugated with a Tri-Color fluorophore in DPBS.
4. Analyzed cells using a dual channel FACScan with a single 488 nm argon laser.
5. Measure GFP fluorescence with a 530 nm band pass filter.
6. Measure Tri-Color fluorescence with a 675 band pass filter.
7. Conduct all experiments at least three times using three identical wells of a 24-well plate each time. see Note 13.

3.6. Flow Cytometry Analysis (GFP)

1. Trypsinize CHO cells and wash twice in Dulbecco's phosphate buffered saline with Ca^{2+} (DPBS).
2. Resuspend in DPBS.
3. Measure GFP fluorescence using a dual channel FACScan or multichannel Caliber flow cytometer equipped with a single 488 nm argon laser.
4. Measure GFP fluorescence with a 530 nm band pass filter. Collect at least 10,000 events per sample.
5. Analyze with CellQuest software or equivalent.
6. Cells can be harvested and analyzed at the time point corresponding to the maximum percentage of cells expressing GFP, 7 h for mRNA transfection and 24 h for DNA transfections.
7. Conduct all experiments at least three times for CHO and NIH3T3 cells, and twice for primary neuronal cells (see Note 13).

3.7. Luciferase Expression Analysis After mRNA Transfection

1. Lyse CHO cells in 200 μl of lysis buffer.
2. Analyze 20 μl of lysate by luciferase assay using an Enhanced Luciferase assay Kit.
3. Measure Quantitative luminescence using a Monolight 2010. see Note 14.

3.8. Optimization of Luciferase Transcript Transfection

Optimization of Luciferase transcript transfection of neuronal cells is similar to CHO procedure.

3.9. In Vivo Luciferase-Expressing mRNA Vector Delivery to Rat Brain (see Note 15)

1. Formulate lipoplexes for in vivo experiments with only mRNA (or DNA as control) and lipid (no OptiMem), in order to minimize injected volume.
2. Anesthetize animal subjects adequately (250–300 g Sprague–Dawley rats in this example) after obtaining an approved animal care protocol (see Note 16).
3. Add lipid to mRNA or DNA to make 3:1 lipid:nucleic acid charge ratio, as above.

   Incubate mixture at 37°C for 30 min prior to delivery.
4. Mount animals in stereotaxic small animal surgery frame.
5. Begin infusion approximately 30 min after incubation of complexes at 37°C.
6. Using sterile techniques, deliver previously optimized formulations at a dose of 50 μg/kg nucleic acid.
7. For 250 g rats, use coordinates of 0.9–1.0 mm posterior and 1.5 mm lateral of midline relative to bregma, at a depth of approximately 3–3.5 mm.

8. Aspirate of CSF to verify intraventricular canula placement.
9. Infuse transfection formulation over 40 min using a syringe infusion pump.
10. Monitor animals closely for signs of discomfort, toxicity, or neurologic injury, although we rarely observe this.

3.10. Tissue Preparation for Luciferase Protein Localization After mRNA Delivery

1. Seven hours after mRNA vector delivery, deeply anesthetize the animal subject.
2. Perfuse through the ascending aorta with iced saline until blood flowing from the transected inferior vena cava is clear.
3. Follow with iced 4% paraformaldehyde in 0.1 *M*, pH 7.4 sodium phosphate-buffered saline (PBS).
4. Dissect out brain and, post fix in paraformaldehyde fixative overnight at 4°C.
5. Incubate in PBS containing 20% glycerol at 4°C.
6. Block brains and cryosection in the coronal plane following standard techniques, beginning approximately 6–7 mm anterior relative to bregma. Ten series of 30-μm serial sections can be collected from each rat brain.

3.11. Diaminobenzidine mmunohistochemistry (see Note 17)

1. Pretreat with 0.1% H_2O_2 for 15 min before washing in modified PBS to eliminate endogenous peroxidase activity and staining artifact.
2. Fix sections in 4% paraformaldehyde.
3. Incubate sections in blocking buffer (0.3% Triton X-100, 3% bovine serum albumin (BSA), 10% normal goat serum (NGS), Ca^{+2} and Mg^{+2}-free PBS for 2 h at room temperature.
4. Dilute primary antibodies in blocking buffer.
5. Incubate sections with antibody solution at 4°C overnight.
6. Wash sections and incubate with the biotin-conjugated secondary antibody, which targets the primary antibody host species, for 1 h at room temperature.
7. Wash sections again and incubate with the tertiary horseradish peroxidase-conjugated probe for 1 h at room temperature.
8. Wash again and incubate in 50 m*M* Tris–HCl at pH 7.6 for 5 min at room temperature.
9. Incubate with 0.5 mg/ml 3,39-DAB with 0.03% H_2O_2 as the peroxidase substrate.
10. Use the following antibodies and dilutions: Primary antibodies: mouse monoclonal neuron specific nuclear protein anti-NeuN (MAB377, 1:50); rabbit polyclonal antiluciferase antibody (CR2029R, 1:50) (see Note 18).

11. Use a microscope with camera mount for photographic documentation of results.
12. Scan film negatives or slides into Photoshop using a slide scanner and Photoshop plug-in at a resolution of 2,700 dpi.
13. Print photographs using Photoshop on a Fuji Pictrography 3000 at 320 dpi, or equivalent.
14. Later experiments were documented on a Nikon Eclipse TS100 Inverted microscope with a high resolution digital camera

4. Notes

1. *Lipids matter*. The protocols described here have been optimized and confirmed over many years and compared against numerous commercially available lipids. We have found (9) that the length of the carbon tails and the symmetry or lack thereof of the carbon tails greatly affects how lipoplexes are taken up and possible also processed by cells. We believe (9) that the asymmetry and size of the linker and hydrocarbon tails of the lipids determines membrane fusogenicity, and furthermore that cell uptake depends not on specific ubiquitous receptors (as it was observed for some viral vectors), but on cell membrane fusion, a slower process. After individual optimization and simultaneous comparison of several commercially available cationic, neutral, dendrimer, and other lipids, we found the novel MLRI cationic lipid (27–29) to consistently perform superior to any other lipid, as evaluated by cell toxicity and efficacy of transfection. MLRI is an asymmetric C(14)-C(12) cationic lipid variant of the highly active cationic lipid DMRI (24). MLRI and similar cationic lipids were described previously by Balasubramaniam et al. (30), Bennett et al. (31, 32), and Felgner et al. (24). Some commercially available and popular lipids are very efficient at cell killing, if one measures toxicity with flow cytometry. MLRI has extremely low toxicity in addition to the high transfection efficacy. MLRI is not easily commercially available, but MLRI can be synthesized by any competent organic chemist, or a specialty lipids manufacturer such as Avanti polar lipids (Alabaster, Alabama) can manufacture it as a specialty order.
2. Prepare mRNA or short RNAs (siRNA, hRNA, micro RNAs) of your desired sequences using standard in vitro transcription and purification techniques.

3. GFP and luciferase reporter transcripts are used for different experiments. Times for maximal luciferase expression can be determined first in luciferase vector lipoplex transfections. These results can then be compared and confirmed using GFP vectors by flow cytometry to determine the maximum percentage of cells expressing GFP, which could be different than the time of maximal luciferase expression in cell lysate, depending on the protein and time course of degradation. Due to the difficulty of isolating and preparing primary neuronal cells, and of more difficult flow analysis of neurons, it may only be possible to perform a limited number of flow cytometry measurements with neuronal cells.
4. Standard cell culture methods are used for cell lines of interest. We present standard methods here for one popular rapidly proliferating cell line, CHOs, and for preparation of a primary neuronal cell culture. NIH3T3 cells, another popular, rapidly growing cell line (ATCC, Rockville, MD) are cultured in 75 cm^2 cell culture flasks with DMEM media (Life Technologies) containing 10% bovine calf serum at 37°C in a 5% CO_2 environment. Split cells 48 h prior to transfection and plated at 60% confluence.
5. Primary neuronal cells are dissected from the cortex of day 17 Sprague–Dawley rat fetuses (as described in ref. 33).
6. Cultures of cortical neuron prepared in this fashion usually contain less than 10% glial cells (33).
7. We used a variety of mRNA transcripts, and all three different plasmids encoding eGFP and luciferase mRNAs used by us gave very similar time courses and levels of expression and are described here.
8. See also the Promega Transfection protocol (34).
9. Although 1 μg each of DNA and RNA are not equal numbers of nucleic acid copies, lipid nucleic acid complexes are formulated based on an equal lipid to nucleic acid charge ratio. Transfast is singly charged per molecule, but not all lipids have a single charge.
10. Promega recommends only 10–15 min at RT.
11. Smaller wells can also be used with a proportional decrease in mass of nucleic acid per well, and a plate reader used for luminescent or other fluorescent analysis technique. Test mRNA in vitro with assay (for our fluorescent or luminescent reporter vectors, we always test with in vitro transfection and Luminometer measurements). We found that in vitro expression of luciferase had to be greater than a threshold of luminescence per μg in vitro or in vivo expression was not sufficiently robust for detection.

12. We found 6–7 h to be the peak using either luciferase cell lysis analysis or GFP flow cytometry results. However, for the DNA vectors, we found that the GFP peak in expression as a percentage of cells expressing GFP occurred slightly (24–36 h) earlier than the peak in luciferase expression (36–48 h). Our luciferase assay requires cell lysis and is a measure of total luciferase protein. Signal from the action of luciferase on luciferin will depend on the concentration and activity of the enzyme present. The half-life of GFP in mammalian cells has been reported to be as long as 26 h (35). The half-life of luciferase in mammalian cells is reported to be from 90 min to 4 h (36–38). Two reviews of the kinetics and modeling of luciferase concentration and activity can be found in refs. (39) and (37). However, we were most interested in the fraction of cells that were transfected rather than the peak in protein expression, which would include intracellular accumulation, and we thus compared flow cytometry results 24 h after DNA delivery and 7 h after RNA delivery based on the peaks in percentage of cells expressing GFP by flow cytometry measurement. We confirmed these peaks in expression with in vivo imaging of both DNA (28) and RNA (data not yet published) luciferase vectors after CNS delivery.
13. Cells in culture vary day to day, even when they are from identical cell source, identical passage number, and identical cell density. For this reason it is best to design experiments so that all cells and plates for a single experiment are plated and grown at the same time.
14. 20 μl of lysate was always sufficient for analysis, but if the signal is either too low or too high the lysate volumes can be adjusted to bring the Luminometer signal into a linear range.
15. Widespread distribution, uptake and expression is possible after nonviral, cationic lipid-mediated gene delivery of mRNA vectors by infusing an optimized formulation of luciferase-encoding (or other nucleic acid sequence of interest) mRNA transcript into the lateral ventricle or into the cisterna magna of rat, mouse, or monkey brain. Perform direct injections using standard techniques that we previously reported (27, 29). We have found that for the cationic lipids that we recommend the in vitro optimizations can be used with only minor further modification in in vivo applications as well. This is not always the case for every lipid. Kariko and colleagues have also demonstrated optimized in vitro and in vivo expression after in vitro mRNA transcription and delivery. Although Kariko et al. have not demonstrated the widespread in vivo expression that Hecker and colleagues have reported (see refs. 29, 40–43), including with mRNA (see refs. 23, 44, 45), Kariko et al. have achieved remarkable protein expression levels after optimization of both transcript and formulation. They have also

investigated vector modifications that both increase immunogenicity (for vaccine applications) or decrease immunogenicity (for clinical use) (46–49), and for some applications the Kariko enhancements may be critical for optimal protein expression after (m)RNA delivery. We previously reported cationic lipid formulations that afford RNA protection from degradation in human CSF for 4–6 h, whereas non-complexed RNA is immediately degraded (27). Combining mRNA vectors with delivery to the CSF offers rapid expression in the CNS and avoids the problem of vascular barriers and viral vector safety issues. These same lipid-mediated lipoplex delivery techniques can be used effectively for siRNA delivery. We verified knockdown of GFP and luciferase expression in vitro, using Transfast and MLRI cationic lipids and similar charge ratios.

16. In vivo delivery to the rat CSF requires approximately 15 min after needle localization into the lateral ventricle or cisterna magna, resulting in a total effective incubation time of 45–60 min. Anesthetize animals ahead of time or simultaneously so that they are ready or very nearly ready for infusion prior to formulation of lipoplexes.
17. Diaminobenzidine (DAB) and secondary fluorescent immunohistochemistry protocols were optimized for expression using multiple DNA and mRNA vectors (27, 29). These optimizations were conducted with no primary and no secondary controls on slide-mounted sections or in 24-well plates. To ensure identical, simultaneous processing free-floating sections in 24-well plates were stained using the ExtrAvidin® peroxidase system. For comparison, experiments were also processed using sections mounted on poly-l-lysine-coated microscope slides.
18. The NeuN antibody was used for comparisons of the number of neurons that can be identified in each section.

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Chapter 6

Light-Induced mRNA Transfection

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Abstract

mRNA-based transfection is an attractive strategy for manipulation of gene expression for gain-of-function studies and therapeutic applications. As a potential therapeutic regulator, mRNA transfection has mainly been hampered by poor delivery strategies, combined with lack of specific targeting to the intended tissue(s) or cells. In this chapter, we describe a protocol for light-induced mRNA transfection into human cancer cell lines with the benefit for time- and site-specific mRNA targeting. Light-induced mRNA transfection is achieved by delivering mRNA molecules into endosomal and lysosomal vesicles. Subsequently, a photosensitizer (PS) localized in the membranes of these vesicles is used to induce damage, resulting in release of mRNA molecules into the cytosol. The main benefit of the strategy proposed is the possibility for protein production from the delivered mRNA in a way that is controllable in a time- and site-specific manner.

Key words: Light-induced transfection, mRNA molecules, PCI, Photosensitizer, Endosomal pathway

1. Introduction

Light-induced mRNA transfection takes advantage of the translational machinery in the cytosol of cells, which in turn translates the transfected mRNA into protein in a time- and site-specific manner induced by light. Synthetically produced mRNA molecules encoding therapeutic proteins can compensate for defective or missing proteins, as well as add new functionality to cells. Besides RNA-based cancer vaccines, it has become recognized that mRNA transfection represents an attractive approach with therapeutic potential, e.g., for expression of tumor-suppressor genes (1). To achieve mRNA transfection, overcoming barriers such as the cellular membrane and entrapment in endosomal vesicles is mandatory to obtain

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a potent biological effect. Nucleic acids have an anionic charge, restricting efficient cellular uptake. This dilemma can be overcome by using synthetic cationic carriers. Both cationic lipids (2–6) and cationic polycations, such as DEAE-dextran (5), poly(l-lysine) (7), dendrimers (8), and polyethylenimine (9) have previously been investigated for mRNA transfection. As most lipid polyplexes are taken up by endocytosis, liberation from these vesicles is necessary to obtain a biological effect. To improve liberation of macromolecules from the endosomal pathway, several endosome-disruptive methods have been investigated, such as Ca^{2+} treatment (10), chloroquine treatment (11), sucrose treatment (12), and endosome-disruptive carriers (13, 14). However, none of these methods have the capacity for time- and site-controlled delivery of mRNA molecules into the cytosol/nucleus of cells. A strategy called photochemical internalization (PCI) (15) is based on illumination of a photosensitizer (PS) that localizes preferably in endosomal and lysosomal membranes. Illumination of the PS initiates an oxidative process, resulting in formation of reactive oxygen species (ROS) that induce damage upon endosomal membranes, resulting in the release of endocytosed material. Importantly, PCI represents a method for targeted delivery of macromolecules, since the biological effect of macromolecules is preferentially activated in only light-exposed areas. In nonexposed areas, macromolecules are predisposed for degradation in the lysosomes. We have in our previous study developed a strategy for light-induced mRNA transfection (16) by combining transfection of mRNA molecules with PCI technology and the protocol for this strategy is described herein.

2. Materials

2.1. Cell Culture

1. All cell lines should be cultured according to recommendations for the specific cell lines. The protocol described here is for adherent cells (see Note 1).
2. Cell growth medium: RPMI-1640 supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μl/mL streptomycin, and 2 mM glutamine. Store at 4°C.

2.2. Preparation of PS Stock Solution (1 mg/mL)

1. PS: disulfonated *meso*-tetraphenylporphine (TPPS2a) with two sulfonate groups on adjacent phenyl rings (Cat. Number T40637, Porphyrin Products, Logan, UT), (see Note 2).
2. 0.1 M NaOH.
3. Phosphate-buffered saline (PBS): 0.2 g/L KH_2PO_4, 8.0 g/L NaCl, 1.15 g/L Na_2HPO_4. Sterilize by filtration and store at 4°C.

4. Dissolve 1 mg of $TPPS_{2a}$ in a small volume (approx. 0.1–0.2 ml) of 0.1 M NaOH.
5. Dilute with PBS to a final volume of 1 mL. The PS's usually dissolve well in this way.
6. Sterilize by filtration, and store at −20°C in small aliquots for up to 6 months. Can be used several times after thawing and freezing (see Note 5).

2.3. mRNA-Branched Polyethylenimine Complexes

All equipment and reagents must be RNase-free.

1. 25 kDa branched polyethylenimine (B-PEI) (Sigma, St. Louis, MO).
2. RNAse-free water.
3. mRNA stock solution (1 μg/μl), stored at −20°C.
4. Transfection agent B-PEI (1 μg/mL) stored at 4°C (see Note 3).
5. RNase-free sterile polypropylene microcentrifuge tubes.
6. RPMI 1640 (without supplements).

2.4. Photochemical Transfection

1. 6-well culture plates.
2. Light-source for excitation of $TPPS_{2a}$ at 435 nm (see Note 4).
3. Cell growth medium and $TPPS_{2a}$ (0.63 μg/mL).
4. RNase-free sterile polypropylene microcentrifuge tubes containing mRNA–B-PEI complexes.

3. Methods

The subsequent protocol is for light-induced mRNA transfection of cells in a 6-well culture plate (well diameter 3.5 cm) when using the cationic 25 kDa B-PEI. Cationic carriers like PEI are excellent carriers for nucleic acids as they have the ability to neutralize and compact anionic charged nucleic acids. Amounts and volumes presented herein are calculated for one such well, but can be adjusted for other cell culture plates, cell numbers, and volumes (see Note 4).

3.1. Preliminary Experiments to Uncover the Appropriate Light-Dose

The light-dose to be used for light-induced mRNA transfection should be found prior to the main experiments. Cell survival as a function of light-dose and should be measured individually for each cell line, type of cell culture plate, and type of light-source used. As a starting point, the light-dose should be adjusted to just below the initiation of cell killing (see Note 4). Cell viability can be measured by one of the common cell viability tests, such as the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) MTS test, trypan blue, or another suitable test.

3.2. Preparation of Adherent Cells for Light-Induced mRNA Transfection

The cells are seeded out 2 days prior to the experiment. In a 6-well culture plate, cells are seeded in 2 mL/well of cell growth medium. The number of cells seeded out per well depends on the cell size and cell growth rate. For 24 h incubation (after light-treatment), cells are usually seeded at 20–40% confluence 2 days prior to incubation with mRNA–B-PEI complexes. Higher and lower cell densities may also be used: therefore the seeding density should be adjusted for every cell line (due to size and grow rate variations) and incubation time.

3.3. Preparation of TPPS$_{2a}$ Working Solution (0.5 μg/ml)

Work in subdued light (see Note 5). Working solution should be freshly made before application to the cells. Add 0.63 μL of TPPS$_{2a}$ stock solution (1 mg/mL) to 0.8 mL of growth medium. This solution is now ready to be applied to the cells.

3.4. Preparation of the mRNA–B-PEI Complexes

The complexes should be prepared just before addition to the cells.

As an example we here make complexes with an N/P ratio of 3 (see Note 6); therefore, the amounts presented below correspond to complexes with an N/P ratio of 3.

1. Prepare the mRNA solution in a separate sterile microcentrifuge tube: 1 μl of mRNA (1 μg/μl) diluted to 100 μL in RPMI 1640 (without supplements). Gently mix the solution by pipetting several times. Do not vortex.
2. Prepare B-PEI solution in a separate sterile microcentrifuge tube: 1 μl of B-PEI stock solution (1 μg/mL) diluted to 100 μL in RPMI 1640 (without supplements). Gently mix the solution by pipetting several times. Do not vortex.
3. Transfer the B-PEI solution into the microcentrifuge tube containing the mRNA solution. The final volume of the mixture is 200 μL. Gently mix the mixture by pipetting several times. Do not vortex.
4. Leave the mixture on the bench at room temperature for 30 min to allow formation of mRNA–B-PEI complexes.
5. Transfer the whole mRNA–B-PEI mixture (200 μL) into the well containing 800 μL growth medium making a total volume of 1 mL.

3.5. Light-Induced mRNA Transfection In Vitro

All the procedures from this point onwards should be performed in subdued light (see Note 5). A simplified experimental scheme is presented in Fig. 1.

1. Seed out the cells 2 days before the experiment (see Subheading 3.2).
2. Remove the growth medium from cells and add 800 μL of growth medium containing 0.63 μg/mL TPPS$_{2a}$ (see Subheading 3.3).

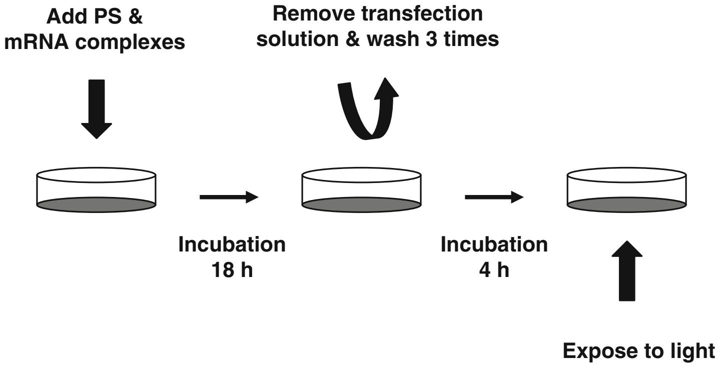


Fig. 1. Experimental scheme.

3. Add 200 μL growth medium containing freshly prepared mRNA–B-PEI mixture (see Subheading 3.4).
4. Incubate overnight, i.e., for 16–18 h (see Note 7) at 37°C in a CO_2 incubator.
5. Remove the medium and wash the cells three times with growth medium to remove $TPPS_{2a}$ on the cell surface.
6. Incubate at 37°C in a CO_2 incubator for the desired time. We recommend 4 h (see Note 8).
7. Expose the cells to light with appropriate dose and wavelength. The optimal light-exposure time depends on the cell line used; the light-dose must therefore be determined separately (see Note 4).
8. Grow the cells for preferred time and analyze the biological effect by elevated protein production from the given mRNA, e.g., by western blotting. Protein production usually peaks between 12 and 24 h after the mRNA is internalized into cytosol (see Note 9).

4. Notes

1. Both adherent cells and non-adherent cells have potential within light-induced mRNA transfection. However, most non-adherent cells are usually harder to transfect. Second, there is a challenge of giving a uniform light-dose to each cell in the well/flask when in suspension. We have only gained successful results when using adherent cells and the protocol described here is therefore for adherent cells.
2. We have here described a strategy for light-induced mRNA transfection based on the use of $TPPS_{2a}$, a PS commonly used

Table 1
An overview over various carriers tested for light-induced mRNA transfection in the OHS cell line. Abbreviations: B-PEI = branched (B) polyethylenimine (PEI), kDa = kilo Dalton

Carriers tested for light-induced mRNA transfection	Protein expression without PCI	Protein expression with PCI
jetPEI™	>60%	>60%
B-PEI 1.8 kDa	0–15%	>60%
B-PEI 25 kDa [16]	0–15%	80–90%

within PCI applications. The $TPPS_{2a}$ is based on the porphyrin skeleton and due to its anionic amphiphilic nature it localizes in the membranes of endocytic vesicles. However, other PS's, localized in the membranes of endocytic vesicles, may also be used. If other PS's than $TPPS_{2a}$ are to be used, it may, however, be necessary to employ other wavelengths, in order to match the spectral characteristics of the specific PS.

3. We have tested a few cationic carriers for light-induced mRNA transfection as they have the ability to neutralize and compact nucleic acid molecules like for example mRNA (see Table 1). Importantly, to obtain light-induced mRNA transfection, the transfection agent must be entrapped in endosomal and lysosomal compartments before light-exposure. Transfection reagents with effective intrinsic fusogenic or endosmolytic activity (e.g., Lipofectamine 2000 and jetPEI) are less suitable for light-directed delivery, as they have a trend to escape endosomal and lysosomal compartments as independent units. However, for some transfection agents, the level of escape from endosomal and lysosomal compartments can be reduced/abolished by lowering the N/P ratio (meaning N > P in the carrier/RNA mix), as shown in our example when using B-PEI. It is therefore necessary to optimize the N/P ratio to obtain a light-directed effect prior to experiments. As a starting point, the N/P ratio should be just above the observable effect for mRNA transfection (when transfecting a fluorescent mRNA), typically a transfection effectiveness of 0–10% positive cells (fluorescence signal) without PCI. To achieve effective transfection (>80%) when combining PCI with PEI/mRNA it is normally necessary to employ an N/P ratio above 2 or 3 (this is dependent

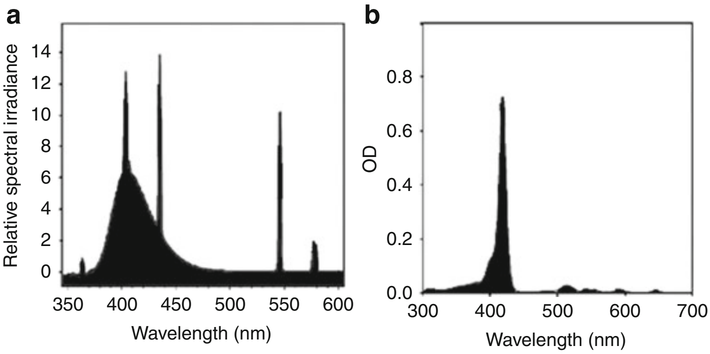


Fig. 2. (**a**) Lamp spectrum of light-source. (**b**) $TPPS_{2a}$ absorption spectrum.

naturally dependent upon the cell line used). For calculation of the N/P ratio (see Note 6).

4. The light-source used in our laboratory is a LumiSource lamp (PCI Biotech) consisting of four light tubes, which deliver blue light with a peak at 435 nm and a fluence rate of 5.1 mW/cm^2. However, other light-sources with appropriate characteristics can also be used. For every cell line to be transfected the illumination time should be adjusted, since the light delivered from different lamps will vary, and also the optimal light-dose will vary between cell lines, cell plate format etc. We routinely do calibration by measuring cell viability using the common cell survival tests MTS, but other cell viability test such as trypan blue or propidium iodide may also be used. In general, a light-dose close to initiation of cell killing would be a good starting point for photochemical transfection. Choosing the light-source, the following points should be taken into consideration:
   (a) The light-source should deliver light of a wavelength suitable for excitation of $TPPS_{2a}$, which has an absorption peak at 435 nm (Fig. 2). It is also an advantage to avoid too much UV light, since UV light may induce cytotoxic effects. We have used the LumiSource lamp from PCI Biotech (Oslo, Norway), which has a filter for excluding UV light. Furthermore, the LumiSource lamp is equipped with a fan to prevent hyperthermia.
   (b) The light source should generate a homogeneous light field in the area where the cells are illuminated.
   (c) Although light has to penetrate the plastic dish before reaching the cell monolayer, the type of plastic dish does not seem to influence the results, but the well format may influence on the recommended illumination time.

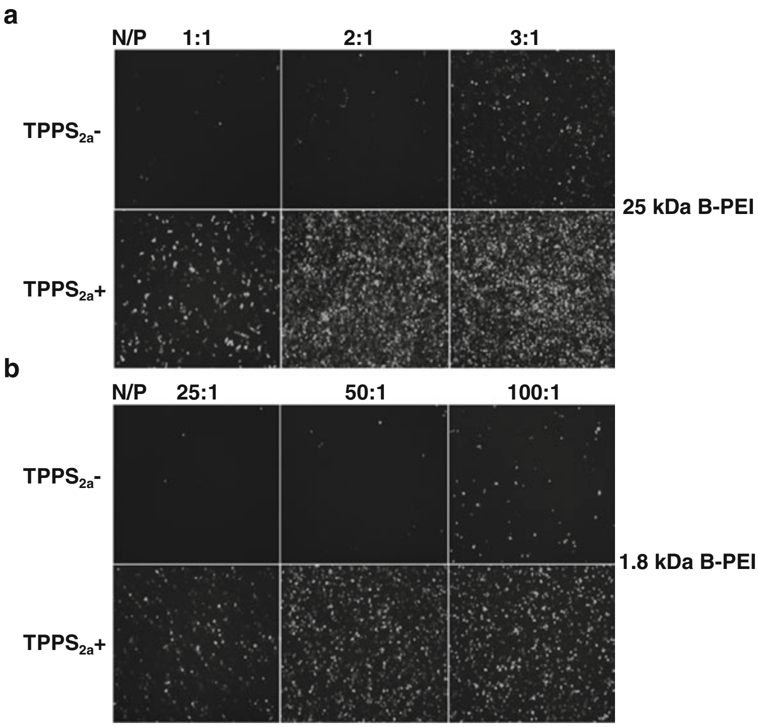


Fig. 3. The effect of 25 kDa (**a**) vs. 1.8 kDa (**b**) B-PEI upon light-induced EGFP mRNA transfection in the OHS cell line. Pictures were taken 24 h after light-exposure without (*upper panel*) or with (*lower panel*) PCI. EGFP expression is shown at N/P ratios of 1:1, 2:1, and 3:1 for 25 kDa B-PEI and 25:1, 50:1, 100:1 for 1.8 kDa B-PEI (*from left to right*).

5. $TPPS_{2a}$ is a relatively photostable PS. However, we recommend protection of the PS solutions from light by aluminum foil to avoid possible light-inducible damage to the PS itself. The PS's may also aggregate after prolonged storage or repeated freezing and thawing. Aggregation will reduce the efficacy of the PS and should be avoided. Usually the color of the stock solution of $TPPS_{2a}$ is dark brown, and change of the color is usually a bad sign indicating aggregation or decomposition of the PS. In this case, the quality of the stock solution should be checked by measuring the phototoxic effect on cells using cell viability tests.

6. Here, we have described the use of the mRNA–B-PEI complexes with a nitrogen–phosphate (N/P) ratio of 3 (see Subheading 3.4). However, the N/P ratio should be optimized for photochemical transfection. In general, using lower molecular weight B-PEI will require an increase in N/P ratio to obtain potent mRNA transfection (see Fig. 3). Different approaches are used to define the exact composition of lipo- and polyplexes, such as the weight/weight (w/w) ratio, the charge ratio, and N/P ratio. We have used the N/P ratio in

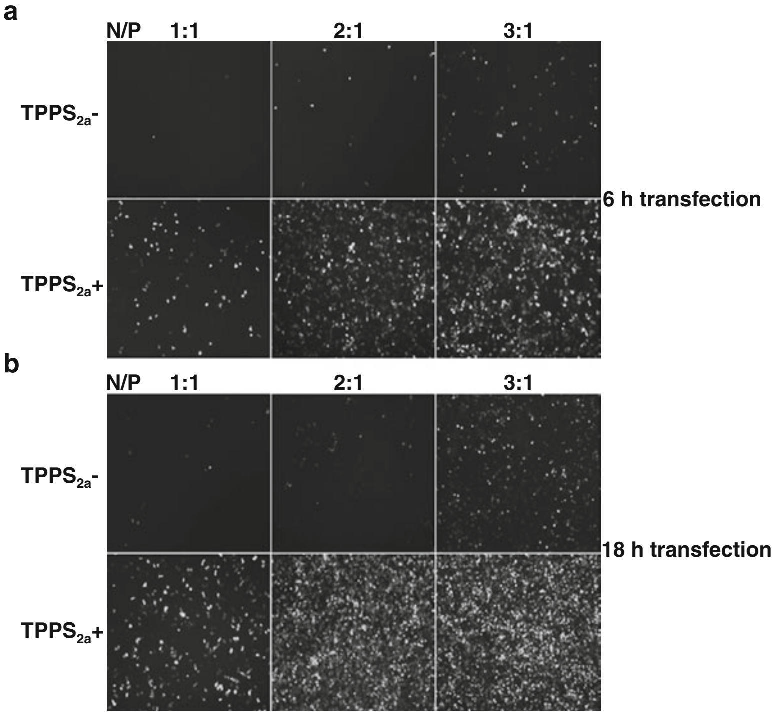


Fig. 4. The effect of 6 h (**a**) vs. 18 h (**b**) EGFP mRNA transfection time upon light-induced mRNA transfection in the OHS cell line. Pictures were taken 24 h after light-exposure without (*upper panel*) or with (*lower panel*) PCI. EGFP expression is shown at N/P ratios of 1:1, 2:1, and 3:1 (*from left to right*).

our work, as this is the most common way to define PEI polyplexes. The N/P ratio is expressed as nitrogen in PEI per phosphate group in the nucleic acid molecule. The N/P ratio of PEI/siRNA polyplexes = [Amount of PEI × Monomer size of siRNA]/[Amount of siRNA × Monomer size of PEI].

An example of N/P calculation:

(a) 0.4 μg PEI with monomer size = 43 g/mol

(b) 1.0 μg mRNA with monomer size of 316.7 g/mol

(c) Using the formula above: $\mathrm{N}/\mathrm{P} = [1 \times 316.7]/[1.0 \times 43]$

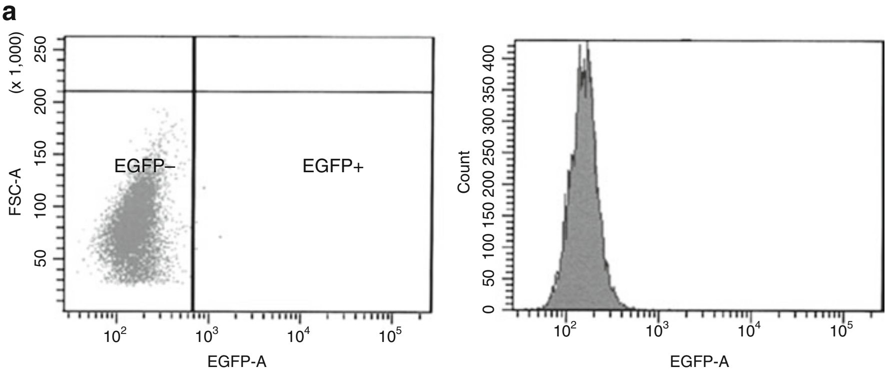
(d) N/P = 3.0

7. To ensure high amounts of mRNA molecules in endosomal vesicles, we always incubate the cells with EGFP mRNA for 18 h before three washing steps. An mRNA transfection time of 18 h was chosen based on microscopy data, giving a higher amount of EGFP positive cells after 18 h of transfection compared to 6 h of transfection (Fig. 4). Our results show that synthetically produced mRNA molecules may be stable when complexed to 25 kDa B-PEI even after ≥22 h in the endosomal pathway as they result in effective protein production after PCI

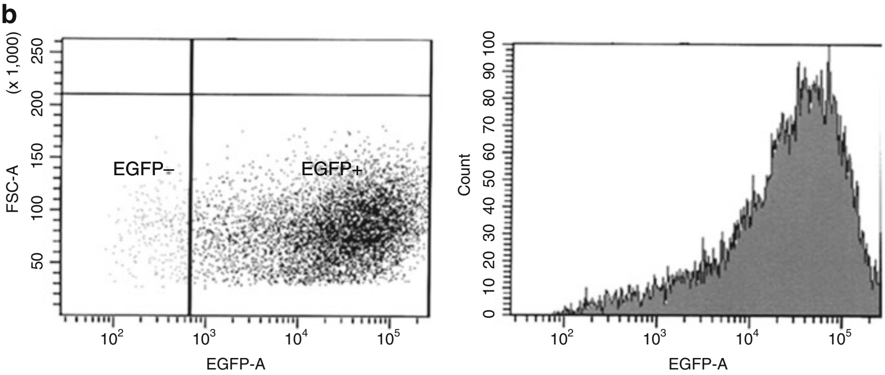
treatment (16). This result implies the capacity of the carrier to protect the nucleic acid from degradation in late endosomes and lysosomes.

8. To reduce damage to the plasma membrane, it is not recommended to expose the cells to light until 4 h after the removal of $TPPS_{2a}$. Therefore, the cells are routinely incubated with the mRNA–B-PEI complexes in $TPPS_{2a}$-free medium for 4 h (see Subheading 3.5, step 5) to ensure that most of the $TPPS_{2a}$ bound to the plasma membrane is washed out or internalized into the cell before irradiation. This ensures that the light-exposure does not induce extensive photochemical damage to the plasma membrane, which is lethal for cells, and that the main effect will be due to induced rupture of endocytic vesicles, which is where $TPPS_{2a}$ localizes. Shorter incubation than 16–18 h with the mRNA–PEI complexes and/or $TPPS_{2a}$ is possible, but the total incubation time in $TPPS_{2a}$-free medium before irradiation should not be much shorter than 4 h. An important consideration is the time needed to remove the main portion of PS from the plasma membrane varies between cell lines. A 4 h incubation in PS-free medium has been found to be suitable in our studies to avoid severe cell toxicity (16–19).

9. A peak in EGFP production between 12 and 24 h has been measured previously after mRNA delivery into cytosol (20). As a starting point, an option is to transfect cells in a 6-well culture plate with 1–3 μg mRNA. The half-life of the protein production will naturally depend upon the stability of the 3 UTR region of the synthetic mRNA given. Furthermore, the half-live of the protein signal will depend upon the stability of the protein produced. The mRNA transfection protocol should preferentially be optimized for efficacy by measuring the effect of protein expression by flow cytometry (when using synthetically produced mRNA molecules encoding a fluorescence protein, e.g., EGFP) prior to a biologically relevant study (see Figs. 3 and 4). The highest transfection efficacy we have obtained when using 25 kDa B-PEI for mRNA transfection combined with PCI is 96.2%, compared to untreated control cells, when measured as EGFP signal by flow cytometry (Fig. 5). When transfecting nonfluorescent mRNA molecules, protein expression after light-induced mRNA expression should be measured by for example western blotting to provide support for correct protein folding after translation of the synthetic delivered mRNA molecule.



	Population	#Events	%Parent	EGFP-A Median	EGFP-A Mean
■	EGFP+	4	0.0	782	898
▨	EGFP−	8,759	99.9	152	160



	Population	#Events	%Parent	EGFP-A Median	EGFP-A Mean
■	EGFP+	6,744	96.2	34,118	50,655
▨	EGFP−	267	3.8	340	355

Fig. 5. Transfection efficacy of 25 kDa B-PEI upon EGFP mRNA delivery in the OHS cell line after PCI-treatment, measured by flow cytometry. (**a**) Untreated control cells showing 0% EGFP positive cells. (**b**) Cells treated with EGFP mRNA–B-PEI complexes and PCI, showing 96.2% EGFP positive cells (compared to untreated control cells).

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Chapter 7

Engineering B Cells with mRNA

Jaewoo Lee, David Boczkowski, and Smita Nair

Abstract

Ex vivo activated B cells are an alternative source of antigen presenting cells (APC). However, the ability of ex vivo activated B cells to function as potent APCs has been a concern especially when compared to dendritic cells (DC). Herein, we introduce a strategy to modulate antigen presentation and immune stimulation functions of activated B cells by co-transfection with multiple mRNAs encoding costimulatory molecules (OX40L, 4-1BBL, and CD80), cytokines (IL-12p35 and IL-12p40) and antigen. These activated B cells modified to express immune stimulatory molecules can be a potent alternative to DCs in immunotherapy.

Key words: B cells, Electroporation, Multiple mRNA co-transfection

1. Introduction

Transfection with mRNAs has been broadly used for delivery of antigen or immune stimulatory molecules into antigen presenting cells (APC) that are able to induce activation of antigen-specific T cells ([1, 2]). Vaccination with APC transfected with mRNA encoding tumor antigens has been approved for human clinical trials for a variety of cancer patients ([1, 2]). Compared to viral transduction and DNA transfection, mRNA transfection has several advantages including safety, efficiency and simplicity of preparation. RNA by itself cannot integrate into the host genome. Thus, gene delivery by transfection with mRNA encoding the corresponding genes can circumvent safety concerns that are associated with DNA- or virus-mediated gene delivery ([3, 4]). Moreover, nonviral DNA transfection is inefficient, particularly in nondividing cells, because of limited trafficking of the DNA to the nucleus

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where transcription occurs while the mRNA transfection strategy avoids the need for entry into the nucleus, which may render the efficiency of mRNA transfection superior to DNA transfection (3, 4). Thus, mRNA transfection is an efficient tool to deliver genetic information into cells and safely modulate cell function.

In this chapter, we introduce a method to introduce a diverse array of immune stimulatory molecules into activated B cells by co-transfection of multiple mRNAs. Ex vivo activated B cells have been shown to be a potential substitute for dendritic cells (DC) as a vaccine adjuvant in immunotherapy (5–13). An ex vivo activated B cell-based immunotherapy has several advantages over a DC-based one, including abundance of cells in peripheral blood, ease of expansion, simple preparation and homogeneity. However, activated B cells have been shown to be less efficient stimulators of T cells than DCs (14–16). Furthermore, B cells regulate DC maturation and function and attenuate Th1 responses (16, 17). Previously, we demonstrated that forced expression of IL-12p70, OX40L, CD80, 4-1BBL, and chicken ovalbumin (OVA) in mouse activated B cells by multiple mRNA co-transfection enhanced proliferation and cytokine production by CD8+ T cells in an antigen specific manner. Furthermore, these RNA-modified activated B cells induced in vitro antigen-specific CTL responses as efficiently as mature DCs (18). Therefore, mRNA transfection is a potent and efficient tool to deliver multiple immune stimulatory molecules into immune or nonimmune cells and endows these cells with the ability to induce adaptive immune responses.

2. Materials

2.1. Solutions and Media

1. All the solutions are prepared with ultrapure deionized (DI) water (H_2O).
2. Buffer for flow cytometry: Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium and magnesium, 2% FBS (Catalog number 16000-044) (Invitrogen, Carlsbad, CA).
3. Cell culture medium for murine B cells: RPMI-1640, 10% heat-inactivated FBS, penicillin, streptomycin, sodium pyruvate (1 mM), minimal essential medium nonessential amino acids (0.1 mM, 100× stock), HEPES, pH 7.4 (10 mM), L-glutamine (2 mM), minimal essential medium amino acids (50× stock), and β-mercaptoethanol (5×10^{-2} mM).
4. Cell suspension medium: Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium and magnesium, 2% FBS, and 1 mM EDTA.
5. Media used for electroporation: Opti-MEM® reduced serum medium (catalog number 51985-034) (Invitrogen).

2.2. Magnetic-Based Primary Mouse Splenic B Cell Isolation Components

1. EasySep™ Mouse B cell enrichment kit (Catalog number 19754) (Stem Cell Technologies, Vancouver, BC, Canada).
2. EasySep® Magnet (Catalog number 18000) (Stem Cell Technologies).
3. Falcon™ 5 ml (12 × 75 mm) polystyrene round-bottom tubes.

2.3. B Cell Activation

1. B-type CpG oligodeoxynucleotide 5′-TCCATGACGTTCCTGATGCT-3′ (CPG ODN 1668) (Catalog number tlrl-1668) (InvivoGen, San Diego, CA).
2. Anti-mouse CD40 antibody (HM40-3) (Cat number 14-0402) (eBioscience, San Diego, CA).
3. B cell activation medium: Cell culture medium supplemented with 1 μM CpG ODN 1668 and 10 μg/ml anti-mouse CD40 antibody.

2.4. Expression Plasmid Vectors

1. pSP73-Sph/mIL-12p35/A64 (18).
2. pSP73-Sph/mIL-12p40/A64 (18).
3. pGEM4Z-mOX40L/A64 (19).
4. pGEM4Z-GFP/A64 (20).

2.5. In Vitro Transcribed RNA Preparation Components

1. Restriction enzyme: Spe I, Not I.
2. DNA purification: QIAquick PCR purification kit (Catalog number 28104) (Qiagen, Valencia, CA).
3. RNA purification: NucleoSpin® RNA Clean-up kit (Catalog number 740948, MACHEREY-NAGEL, Bethlehem, PA).
4. In vitro transcription reaction: MEGAscript® kit (Catalog number AM1333) (Ambion, Austin, TX).
5. DNase: Turbo™ DNase (Catalog number AM2238) (Ambion).
6. 5′ Posttranscriptional modification reagents:
    - (a) 5′ Capping: m7G(5′)ppp(5′)G cap analog (Catalog number S1404) (NEB).
    - (b) 3′ Polyadenylation: poly(A) tailing kit (Catalog number AM1350) (Ambion).

2.6. Denaturing Formaldehyde Agarose Gel Electrophoresis Components

1. Buffers.
    - (a) 10× Formaldehyde gel running buffer: NorthernMax® 10× running buffer (Catalog number AM8671) (Ambion).
    - (b) 2× RNA loading dye (Catalog number R0641) (Fermentas, Glen Burnie, MD): 95% formamide, 0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 0.025% ethidium bromide, 0.5 mM EDTA.

2. 1.2% Formaldehyde agarose gel:
   (a) Heat 1.2 g agarose in 90 ml water in a microwave.
   (b) Cool in a 65°C water bath.
   (c) Add 10 ml NorthernMax® 10× denaturing gel buffer (Catalog number AM8676) (Ambion). Mix well. Pour gel in a fume hood.

2.7. Native Agarose Gel Electrophoresis Components

1. Buffers.
   (a) Stock Buffer: 10× Tris-Borate-EDTA (TBE) buffer: 890 mM Tris base, 890 mM boric acid and 20 mM Na-EDTA (pH 8.0) in water.
   (b) Native agarose gel running buffer: 0.5× TBE.
   (c) 2× RNA loading dye (Catalog number R0641) (Fermentas).
2. 1.2% native agarose gel:
   (a) Melt 1.2 g agarose in 100 ml 0.5× TBE using a microwave.
   (b) Add 0.5 μg/ml ethidium bromide.
   (c) Pour onto a gel-casting tray.
   (d) Cool at room temperature until gel hardens.
3. 0.24–9.5 kb RNA ladder (Catalog number 15620-016) (Invitrogen).

2.8. Electroporation Supplies

1. Electro Square Porator ECM 830 (BTX, San Diego, CA).
2. 2 mm gap cuvette.

3. Methods

3.1. Generation of In Vitro Transcribed mRNA with 5′ Cap and 3′ Poly(A) Tail

1. Digest the expression plasmid vectors (Fig. 1) with the restriction enzyme Spe I.
2. Purify the linearized transcription vector using a QIAquick PCR purification kit following the manufacturer's instructions and using buffers that are included in the kit:
   (a) Add 5 volumes of Buffer PBI (included in QIAquick PCR purification kit) to 1 volume of the PCR sample and mix.
   (b) Place a QIAquick spin column in a 2 ml collection tube.
   (c) To bind DNA, apply the sample to the QIAquick column and centrifuge at 16,000 × *g* for 30–60 s.
   (d) Discard flow-through. Place the QIAquick column back into the same tube.

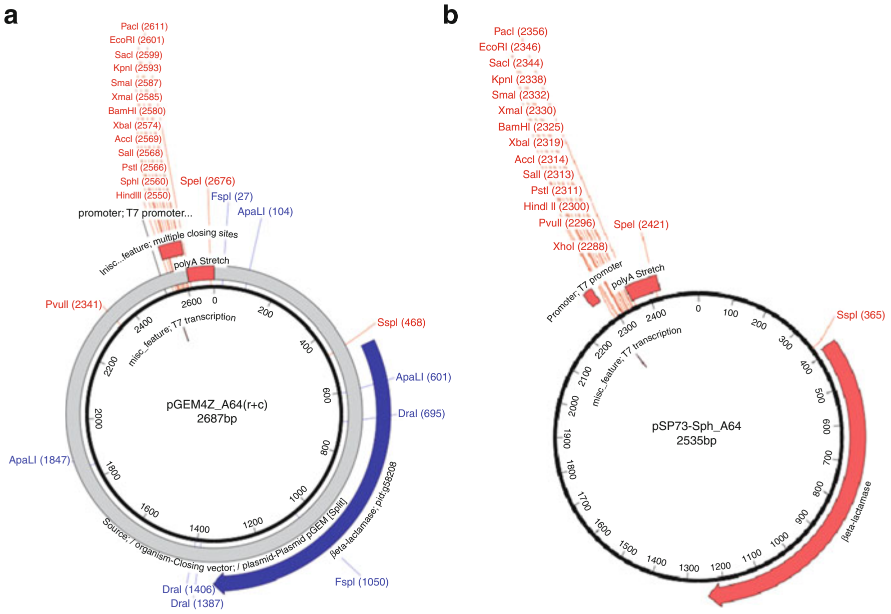

Fig. 1. Transcription vector pGEM4Z_A64 and pSP73-Sph_A64.

(e) To wash add 0.75 ml Buffer PE (included in QIAquick PCR purification kit) to the QIAquick column and centrifuge at 16,000×*g* for 30–60 s.

(f) Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for 1 min at maximum speed.

(g) Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

(h) To elute DNA, add 30–50 μl nuclease-free H_2O to the center of the QIAquick membrane and centrifuge at 16,000×*g* for 1 min.

(i) Determine the quantity and purity of DNAs by reading optical density (OD) at wavelengths of 260 nm (OD_{260}) and 280 nm (OD_{280}) using a spectrophotometer (see Note 1).

3. Prepare in vitro transcription reaction mixture containing 7.5 mM ATP, 7.5 mM CTP, 7.5 mM UTP, 1.5 mM GTP, T7 polymerase mix, 1× reaction buffer, 1 μg linearized plasmid and 6 mM m7G(5′)ppp(5′)G cap in a 20 μl total reaction volume. Control in vitro reaction mixture includes control template DNA pTRI-Xef instead of target expression plasmid vectors. The pTRI-Xef control template is included in the MEGAscript® kit.
4. Incubate reaction mixture for 3–4 h at 37°C.
5. Digest template plasmid by adding two units of DNase.

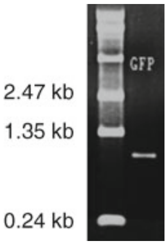

Fig. 2. Gel electrophoresis of in vitro transcribed RNA. One μg of GFP RNA (approximately 0.85 kb) and 3 μg of RNA ladder run at 100 V for 1 h on a 1% native agarose gel.

6. Incubate for 15 min at 37°C.
7. Add poly(A) tail reagents including 36 μl nuclease-free water, 10 μl 25 mM $MnCl_2$, 10 μl 10 mM ATP, 4 μl *E. coli* Poly(A) Polymerase (*E*-PAP) and 20 μl 5× *E*-PAP Buffer included in the Poly(A) tailing kit (see Note 2).
8. Incubate for 1 h at 37°C.
9. Isolate RNA transcripts using NucleoSpin® RNA Clean-up kit.
10. Determine the quantity and purity of transcripts by reading OD_{260} and OD_{280} (see Note 1).
11. Analyze integrity of RNA using a native agarose gel electrophoresis (21) (see Note 3).

    Figure 2 provides examples of analysis of in vitro transcribed green fluorescence protein (GFP) mRNA by native gel electrophoresis.

3.2. Reprogramming B Cells by Transfection with Multiple mRNAs

1. Isolate primary B cells from a mouse spleen by a magnetic-based negative selection using the EasySep™ Mouse B cell enrichment kit following the manufacturer's instructions:
   (a) Physically dissociate a spleen in DPBS with 2% FBS by rubbing the spleen back and forth over a wire mesh.
   (b) Filter the cell suspension through a 70-μm nylon filter into a 50 ml conical tube.
   (c) Centrifuge at 300 × *g* for 10 min.
   (d) Resuspend at 1×10^8 cells/ml in cell suspension medium.
   (e) Place up to 2 ml of cells in a 5 ml polystyrene tube.
   (f) Add the rat serum at 50 μl/ml.
   (g) Add EasySep mouse B cell enrichment antibody cocktail at 50 μl/ml.
   (h) Mix well and incubate in refrigerator for 15 min.
   (i) Add EasySep biotin selection cocktail at 100 μl/ml and incubate in refrigerator for 15 min.

   - (j) Add EasySep magnetic particles at 100 μl/ml and incubate in refrigerator for 5 min.
   - (k) Add cell suspension medium up to a total volume of 2.5 ml.
   - (l) Mix the cells by gently pipetting.
   - (m) Place the tube into the EasySep magnet and incubate for 5 min at room temperature.
   - (n) Pick and invert the magnet and tube in one continuous motion.
   - (o) Collect unbound fraction (i.e., B cells) in a new 5 ml polystyrene tube.
   - (p) Centrifuge at 300 × *g* for 10 min.
   - (q) Resuspend cells at 1×10^6 cells/ml in B cell culture medium.

2. Activate primary B cells by overnight culture in B cell activation medium (see Note 4). Transfer B cells into a 15 ml conical centrifuge tube.
3. Centrifuge at 300 × *g* for 5 min.
4. Discard the supernatant.
5. Resuspend cells in 10 ml Opti-MEM.
6. Centrifuge for 300 × *g* for 5 min.
7. Discard the supernatant.
8. Resuspend cells in Opti-MEM at final cell concentration of 1×10^7 B cells/ml.
9. Transfer 200 μl of cell suspension into a sterile 0.6 ml microcentrifuge tube.
10. Add the mixture of in vitro transcribed OX40L, 4-1BBL, IL-12p35, IL-12p40 and GFP mRNAs (up to 35 μg total amount of RNA) to activated B cells.
11. Mix and incubate at room temperature for 5 min.
12. Transfer cells into a 2 mm electroporation cuvette.
13. Electroporate at 340 V for 500 μs using an Electro Square Porator ECM 830 (see Note 5).
14. Transfer cells into a 60 mm petri dish containing 3 ml of prewarmed B cell culture medium using a sterile disposable transfer pipette.
15. Harvest cells and supernatants at 24 h after electroporation for analysis of membrane, intracellular and extracellular proteins encoded in transfected mRNAs. Figure 3 demonstrates the expression of mouse OX40L (membrane), mouse IL-12p70 (a heterodimer of IL-12p35 and IL-12p40) (extracellular), and GFP (intracellular) proteins.

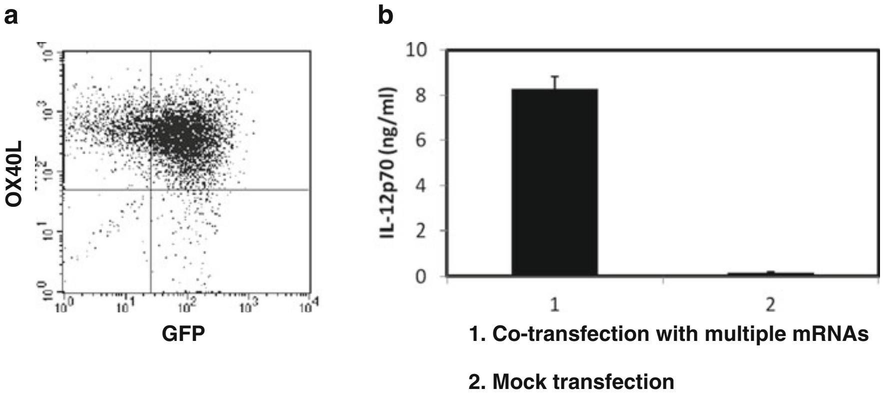

Fig. 3. Simultaneous electroporation of multiple in vitro transcribed RNAs into activated mouse B cells. Primary B cells isolated from a mouse spleen were activated overnight with CpG ODN 1668 and agonistic anti-CD40 antibody. Activated B cells were electroporated with mixture of OX40L, IL-12p35, IL-12p40, and GFP RNAs (5 μg each). At 24 h after transfection, (**a**) surface expression of OX40L and intracellular expression of GFP were analyzed by flow cytometry after staining with phycoerythrin (PE)-conjugated anti-mouse OX40L antibody, and (**b**) secreted cytokine IL-12p70, a heterodimer of IL-12p35 and IL-12p40, was measured using mouse IL-12p70 enzyme-linked immunosorbent assay (ELISA).

4. Notes

1. OD_{260} reading of 1.0 is equivalent to approximately 50 ng/μl double-stranded DNAs and 40 ng/μl RNA transcripts. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 to 2.0. Single in vitro transcription reaction generally produces 40–60 μg of RNA transcripts from 1 μg DNA template.
2. A poly(A) tail reaction adds additional ≥150 base poly(A) tail to RNA transcripts.
3. Loading of in vitro transcribed RNAs heated for 5 min at 65°C with the RNA loading buffer can be run on either denaturing or native agarose gel. Native gel electrophoresis is sufficient to judge the integrity and overall quality of mRNA as compared to denaturing gel electrophoresis. An example is provided in Fig. 2. It also demonstrates the expression of GFP proteins in cells electroporated with in vitro transcribed GFP mRNA. If transcription reaction products produce a smear or run as more than one band, or as smaller than expected when they run on a gel the DNA template is probably contaminated with RNase and the transcription reaction may be prematurely terminated. Thus, the quality of RNA transcripts is unacceptable.
4. Prior-activation of primary B cells by incubation with CpG ODN 1668 and anti-CD40 antibody is required for the successful transfection with mRNAs. However, other primary cell

types (e.g., skin fibroblasts and bone marrow cells) do not need pre-activation for efficient mRNA transfection. It is still unclear why resting B cells are resistant to transfection with mRNAs.

5. Two consecutive electroporations increase transfection efficiency but significantly reduces cell viability after transfection.

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Chapter 8

Programming Human Dendritic Cells with mRNA

Jaewoo Lee, David Boczkowski, and Smita Nair

Abstract

Transfecting with in vitro transcribed, protein-encoding mRNA is a simple yet effective method to express high levels of the desired RNA-encoded proteins in primary cells. Cells can be transfected with antigen-encoding mRNA, which is translated into protein and is processed by the cellular antigen-processing pathway to generate antigen-presenting cells. Another elegant and increasingly popular application is to transfect cells with mRNA that encodes immune modulating molecules (cytokines, chemokines, toll-like receptors (TLRs), immune receptor ligands, immune receptor targeting antibodies) which, when translated into protein, can program cell behavior and/or function. In this chapter we describe an efficient method to deliver mRNA into human dendritic cells (DCs) by electroporation. This is currently the method of choice to deliver mRNA into antigen-presenting cells for generating vaccines for cancer immunotherapy.

Key words: Dendritic cells, Electroporation, RNA-based programming, RNA transfection

1. Introduction

Transfection of human dendritic cells (DCs) (1, 2) with mRNA has multiple applications (3, 4). Most commonly it has been used to deliver antigen (viral or tumor-associated) to DCs to generate antigen-presenting cells ex vivo (3, 5). In addition, many recent studies have successfully used RNA to program or modulate cells to enable specific functions or behavior in the transfected cells (6–16), such as DCs, by transfecting or rather co-transfecting with RNA that encodes immune modulatory molecules, with the objective of inducing robust and effective immune responses. Vaccination with DCs transfected with mRNA encoding tumor antigens has now been translated into clinical trials in cancer patients (3, 4). Compared to other methods used for gene delivery, such as transduction with

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viral vectors and DNA transfection, mRNA transfection is safe, efficient and simple.

In this chapter we describe in detail the procedure for delivering mRNA into DCs. The procedure outlined below entails electroporating mRNA into cells (3, 4, 7, 17). Although much of our preliminary work has focused on demonstrating the utility of RNA-electroporated DCs as cancer vaccines (18–22), recent studies have highlighted the ability to use RNA to engineer DCs to deliver antibodies locally, to enhance the antigen-presenting function of DCs and to drive DC maturation (6, 7, 10, 12, 14). Using antibody-secreting DCs is currently being evaluated in ongoing clinical studies in metastatic melanoma patients (clinicaltrials.gov, NCT01216436).

Lee et al. have also demonstrated the effectiveness of RNA-based programming by delivering multiple mRNAs simultaneously into activated B cells (13). Pruitt and colleagues have successfully used electroporation to modulate the antigen processing mechanism in DCs by electroporating immunoproteasome-targeting siRNA into DC progenitors (CD14+ monocytes) (11). This concept has been translated into a phase I cancer immunotherapy clinical trial in metastatic melanoma patients (clinicaltrials.gov, NCT00672542).

RNA-based programming of cells is a convenient, robust and clinically relevant approach to modify cells to develop effective cell-based therapies. The procedures described below are easily adapted to other cell types, including T cells (for adoptive T cell therapy) and progenitor cells (for RNA-based reprogramming).

2. Materials

2.1. Dendritic Cell Generation

1. Peripheral blood mononuclear cells (PBMCs) (see Notes 1–3).
2. Cellstripper, enzyme-free cell dissociation buffer (Catalog Number 25-056-CI) (Cellgro, Mediatech Inc, Manassas, VA).
3. Cell culture medium for human DC generation: AIM-V medium (Catalog Number 12055-083) (Invitrogen), 800 U/ml human GM-CSF (Leukine® (sargramostim), a recombinant form of granulocyte-macrophage colony-stimulating factor [GM-CSF], 500 U/ml human IL-4. See Note 4.
4. Media used for electroporation: Opti-MEM® reduced serum medium (Catalog Number 51985-034) (Invitrogen).
5. Media used for DC maturation: AIM-V media containing GM-CSF (800 U/ml), IL-4 (500 U/ml), TNF-α (5 ng/ml), IL-1ß (5 ng/ml), IL-6 (150 ng/ml), and prostaglandin E2 (PGE_2) (1 μg/ml).

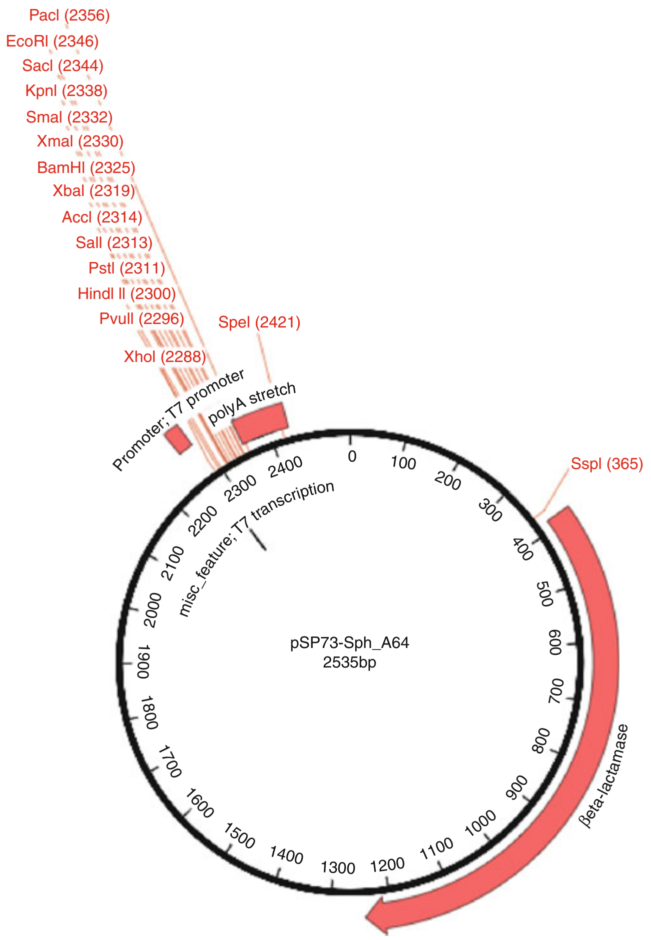

Fig. 1. Transcription vector pSP73-Sph_A64.

6. 50 ml polypropylene tubes.
7. T-75 or T-150 tissue culture flasks.

2.2. Generation of In Vitro Transcribed RNA

1. Transcription vector pSP73-Sph_A64 (Fig. 1).
2. Vector containing green fluorescent protein (GFP) (pSP73-Sph/GFP_A64) (22).
3. Vector containing human glucocorticoid-induced TNFR-related protein-ligand (GITR-L) fusion protein (pSP73-Sph/hIg-hGITR-L_A64) (14).
4. Vector containing anti-human cytotoxic T lymphocyte antigen 4 (CTLA-4) light chain (pSP73-Sph/anti-CTLA-4-light_A64) (14).
5. Vector containing anti-human CTLA-4 heavy chain (pSP73-Sph/anti-CTLA-4-heavy_A64) (14).

6. QIAquick PCR purification kit (Catalog Number 28104) (Qiagen, Valencia, CA).
7. Restriction enzyme Spe I.
8. mMESSAGE mMACHINE® T7 Ultra kit (Catalog Number AM1345) (Ambion, Austin, TX).
9. Turbo™ DNase (Included in mMESSAGE mMACHINE® T7 Ultra kit).
10. Poly(A) tailing kit for 3′ polyadenylation (Included in mMESSAGE mMACHINE® T7 Ultra kit).
11. RNeasy Mini kit (Catalog Number 74104) (Qiagen).
12. In vitro transcription reaction mixture: 7.5 mM ATP, 7.5 mM CTP, 7.5 mM UTP, 1.5 mM GTP, T7 polymerase mix, 1× reaction buffer (included in mMESSAGE mMACHINE® T7 Ultra kit), 1 μg linearized plasmid, 6 mM 3′-0-Me-m^7G(5′)ppp(5′)G anti-reverse cap analog (ARCA) (see Note 5).

2.3. Native Agarose Gel Electrophoresis Components

1. Stock Buffer: 10× Tris-Borate-EDTA (TBE) buffer: 890 mM Tris base, 890 mM boric acid, and 20 mM sodium-EDTA (pH 8.0) in water.
2. Native agarose gel electrophoresis buffer: 0.5× TBE.
3. Loading Buffer (Included in mMESSAGE mMACHINE® T7 Ultra kit).
4. Ambion® RNA Millennium™ Markers (Catalog Number AM7150) (Ambion).

2.4. 1.2% Native Agarose Gel

1. Melt 1.2 g agarose in 100 ml 0.5× TBE using a microwave.
2. Add 0.5 μg/ml ethidium bromide.
3. Pour onto a gel-casting tray.
4. Cool at room temperature until gel hardens.

2.5. Electroporation Supplies

1. Electro Square Porator ECM 830 (BTX, San Diego, CA, http://www.btxonline.com/).
2. 2 mm gap cuvette (VWR) (see Note 6).
3. 4 mm gap cuvette (VWR).
4. Pipettors: P-1000, P-200 and P-20.

2.6. Flow Cytometry Analysis (Also Called Fluorescence Activated Cell Sorting)

1. Fluorescence Activated Cell Sorting (FACS) Buffer: 1× Dulbecco's PBS (Ca^{2+} and Mg^{2+} free) with 2% fetal calf serum (FCS).
2. Anti-human CD357 (glucocorticoid-induced TNFR-related protein [GITR]) conjugated to allophycocyanin (APC) (Catalog Number 17-5875) (eBioscience, San Diego, CA).

3. Anti-human CD152 (cytotoxic T lymphocyte antigen 4 [CTLA-4]) conjugated to APC (Catalog Number 17-1522) (eBioscience).
4. Chinese hamster ovary (CHO) cells expressing GITR (CHO-GITR) to detect GITR-ligand (L) fusion protein in supernatant.
5. Chinese hamster ovary (CHO) cells expressing GITR (CHO-CTLA-4) to detect anti-CTLA-4 in supernatant.
6. Biotinylated anti-human IgG for indirect FACS analysis (Catalog Number 13-4998) (eBioscience).
7. Streptavidin-APC (Catalog Number 17-4317) (eBioscience).
8. 12×75 mm polystyrene Falcon tubes.
9. Formaldehyde 10% Ultrapure.
10. Becton Dickinson FACSCalibur.

3. Methods

3.1. Generation of In Vitro Transcribed mRNA with 5′ Cap and 3′ Poly(A) Tail

1. Digest the transcription vector pSP73-Sph/A64 (Fig. 1) containing target complementary DNA (cDNA) with the restriction enzyme Spe I.
2. Purify the linearized transcription vector using a QIAquick PCR purification kit.
3. Prepare in vitro transcription reaction mixture in a 20 μl total reaction volume.
4. Incubate reaction mixture for 3–4 h at 37°C.
5. Add 2 units of Turbo™ DNase.
6. Incubate for 15 min at 37°C.
7. Add poly(A) tailing reaction mixture: 36 μl nuclease-free water, 10 μl 25 mM $MnCl_2$, 10 μl 10 mM ATP, 4 μl *E. coli* Poly(A) Polymerase (*E*-PAP) and 20 μl 5× *E*-PAP buffer included in the Poly(A) tailing kit (see Note 7).
8. Incubate for 1 h at 37°C.
9. Isolate RNA transcripts using RNeasy Mini kit.
10. Analyze purity and determine concentration of transcripts by measuring the absorbance at 260 nm (A260) and 280 nm (A280) in a spectrophotometer (see Note 8).
11. Analyze integrity of RNA using native agarose gel electrophoresis (see Note 9).
12. Take an aliquot containing 200–500 ng of mRNA and mix with equal volume of loading buffer.

13. Mix the sample and heat at 65°C for 5 min. Chill mRNA sample on ice for 2–3 min.
14. Load mRNA sample into a well in the 1.2% agarose gel that has been pre-submerged in electrophoresis buffer in an electrophoresis tank.
15. In an adjacent well load a sample of the RNA Millennium™ Marker.
16. Connect the electrodes of the electrophoresis apparatus.
17. Turn on the power supply, and run the gel at 7 V/cm measured between the electrodes until the dye has migrated approximately 2/3 of the way through the gel.
18. Visualize gel on UV transilluminator.

3.2. Generation of Human DCs

1. Resuspend PBMCs at a concentration of 2×10^8 cells in 30 ml of AIM-V medium in a 50 ml polypropylene conical tube.
2. Transfer the PBMC cell solution to a T-150 flask (see Note 10).
3. Place the T-150 flask in a humidified incubator at 37°C, 5% CO_2.
4. Incubate for 1–2 h to allow the monocytes to adhere (see Note 11).
5. Dislodge the non-adherent cells by first rocking the flask from side to side and then firmly tapping the flask.
6. Harvest the medium containing the non-adherent cells and set aside on ice (see Note 12).
7. Ensure that all non-adherent cells have been removed by aspirating off all traces of AIM-V media in the flask.
8. Immediately replenish the adherent cells with 30 ml of cell culture medium for human DC generation.
9. Incubate in humidified incubator at 37°C, 5% CO_2 for 7 days.
10. Harvest the DCs on day 7 by tapping the flask firmly with the palm of your hand to dislodge all the loosely adherent DC clusters (see Note 13).
11. Pipette the media containing the dislodged DCs and collect the cells in a 50 ml polypropylene tube (tube A). Set the cells on ice. Supernatant harvested from this tube will be used later to generate conditioned media for DC maturation (see Note 14).
12. Wash the adherent DCs in the flask with 10 ml PBS to remove traces of media and collect the PBS wash into a separate 50 ml polypropylene tube (tube B).
13. To harvest the adherent DCs, add 20 ml of cellstripper (enzyme-free dissociation buffer) and incubate at 37°C for 15–20 min.

14. Tap the flask again and harvest cells by vigorous pipetting. Add these DCs to the tube that contains the PBS wash (tube B).
15. Centrifuge the tubes A and B (each containing 30 ml of solution) at 300 × *g* for 7–8 min at 4°C.
16. Save the supernatant from tube A to make conditioned media.
17. Combine all the DCs by resuspending in 30 ml of PBS and centrifuge at 300 × *g* for 7–8 min at 4°C.
18. Resuspend DCs in the desired media and count cells for number and viability. These are immature DCs.
19. Use immature DCs for RNA electroporation and freeze excess DCs for eventual use (see Note 15).

3.3. Programming DCs Using RNA Electroporation

1. Resuspend the DCs in 10 ml of Opti-MEM and count the cells.
2. Add 30–40 ml of Opti-MEM and wash the cells to remove all traces of DC generation media.
3. Centrifuge DCs at 300 × *g* for 7–8 min.
4. Turn the BTX electroporator on and set it at a voltage of 300 V and a time of 500 μs (microseconds).
5. Aspirate off all media and gently loosen the pellet by tapping on the tube.
6. Resuspend DCs in Opti-MEM gently using a P-1000 pipettor to attain a final concentration of 25–30 × 10^6 DCs per ml (see Note 16).
7. Transfer 100–200 μl of the DCs into a 2 mm electroporation cuvette (see Note 17).
8. Add RNA to the cuvette (see Note 18 for information on amount of RNA) and mix gently but thoroughly with a P-20 pipettor.
9. Gently tap the cuvette 1–2 times to further mix the RNA and cells (see Note 19). Place the cuvette cap back on the cuvette.
10. Place the cuvette in the electroporation stand. The metal plates of the cuvette should be touching the metal plates of the electroporation stand. Tighten the plates of the electroporation cuvettes so that the cuvette cannot move in the stand.
11. Close the lid of the electroporation stand and check the leads to the electroporator to ensure that they are fully connected (see Note 20).
12. Check that the settings are at 300 V and 500 μs. After you press start, the electroporator will display “charging” then “pulsing” when it is sending current through the cuvette.

Then a read out will display of the actual voltage and time that occurred. These numbers vary slightly during electroporations and can be recorded if needed.

13. Upon completion of electroporation, transfer the DCs into media that has been supplemented with cytokines (see Note 21). This should be done by gently siphoning the cells out of the cuvette with the transfer pipette provided and then adding the cells into media with cytokines (see Note 22). If required, use the transfer pipette to pull up some media and wash the cuvettes as some cells will still remain in the cuvette. When performing multiple electroporations keep DCs on ice (see Note 23).
14. Transfected DCs are incubated at 37°C, 5% CO_2 for 8–18 h. DC concentration is maintained at 10^6/ml and DCs are maintained in maturation media.

3.4. FACS Analysis to Monitor Transfection Efficiency

1. Resuspend cells (DCs transfected with GFP RNA, CHO-GITR, or CHO-CTLA-4) at $2–5 \times 10^6$ cells/ml ($2–5 \times 10^5$ cells in 100 μl) in cold FACS buffer (see Note 24).
2. Add 100 μl of the cell suspension to 12 × 75 mm tubes (see Note 25).
3. Add the appropriate amount of monoclonal antibody directly into the cell suspension at the bottom of the tube (see Notes 26 and 27).
4. Vortex gently and incubate for 20–30 min at 4°C (or on ice) in the dark.
5. If using fluorochrome-conjugated antibody (direct staining procedure), wash once with 3 ml of FACS buffer, centrifuge at 300 × *g* for 5 min (see Note 28). Maintain the cells at 4°C (or on ice) prior to acquisition and analysis on the flow cytometer.
6. For indirect staining procedure (as illustrated in Fig. 2c, d), wash cells once with 3 ml of FACS buffer and resuspend cells in 100 μl of FACS buffer. Add biotinylated anti-human IgG secondary antibody directly into the cells.
7. Vortex briefly and incubate for 20 min at 4°C in the dark.
8. Wash once with 3 ml of buffer, centrifuge at 300 × *g* for 5 min.
9. Resuspend cells in 100 μl of FACS buffer. Add streptavidin-APC (fluorochrome) directly into the cells.
10. Wash once with 3 ml of FACS buffer, centrifuge at 300 × *g* for 5 min.

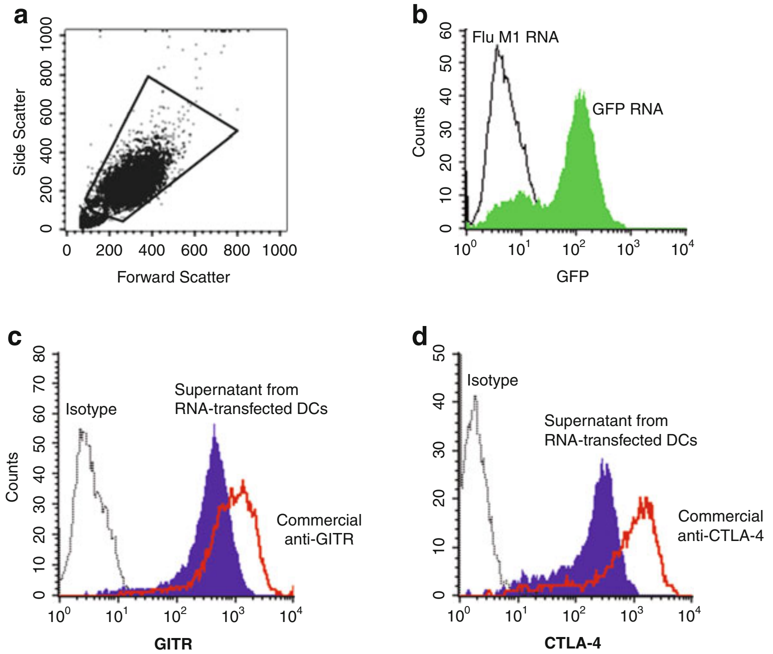

Fig. 2. Electroporating dendritic cells (DCs) with mRNA. Antigen encoding mRNA is used at 4 μg/10^6 DCs. RNA encoding immunostimulatory molecules is used at 10 μg/10^6 DCs. For antibody-encoding mRNA transfections, 10^6 cells are electroporated with 10 μg of heavy (H) chain antibody mRNA and 5 μg of light (L) chain antibody mRNA. Cloning details are provided in our recent publication (6, 14). (**a**) Scatter profile of GFP mRNA-electroporated and matured DCs is depicted. (**b**) GFP expression in DCs that were electroporated with GFP mRNA followed by maturation. DCs electroporated with Flu matrix protein (M1) RNA (25) were used as controls. A FACSCalibur flow cytometer (BD Biosciences) was used to analyze cells. (**c** and **d**) DCs were transfected with mRNA encoding human GITR-L fusion protein (FP) or anti-CTLA-4 H+L chain. The supernatant was harvested after 20 h and concentrated by centrifugation through a 50 kDa cut-off filter (65–70-fold concentrated). The DC supernatant concentrate (20 μl) was incubated with CHO cells expressing CTLA-4 (CHO-CTLA-4) or GITR (CHO-GITR). The cells were then incubated with biotinylated anti-human IgG followed by streptavidin-APC (eBioscience). Commercially available antibodies were used as a positive control. (**c**) Supernatant from DCs transfected with GITR-L fusion protein-encoding mRNA incubated with CHO-GITR cells. (**d**) Supernatant from DCs transfected with anti-CTLA-4-encoding mRNA incubated with CHO-CTLA-4 cells.

11. Maintain the cells at 4°C (or on ice) prior to acquisition and analysis on the flow cytometer. Figure 2a illustrates the scatter profile of RNA-electroporated human DCs following overnight incubation in maturation cytokine cocktail containing media. Figure 2 provides examples of protein expression by human DCs following electroporation with green fluorescent protein (GFP) mRNA (Fig. 2b), soluble GITR-ligand (L) fusion protein mRNA (Fig. 2c) and anti-CTLA-4 encoding heavy (H) and light (L) chain mRNA (Fig. 2d).
12. If cells will be maintained for over 4–6 h and to allow flexibility for analysis of samples on the flow cytometer the cells should be fixed with 1% formaldehyde in FACS buffer (see Notes 29 and 30).

4. Notes

1. Appropriate precautions must be taken when handling human cells, particularly if from unknown, untested donors. All work must strictly adhere to the Institutional guidelines and all protocols required for such work must be approved before any work with human cells is initiated. Biosafety practices must be followed. All procedures use sterile tissue culture techniques with sterile solutions and equipment in biosafety containment hoods.
2. Human peripheral blood mononuclear cells (PBMCs) are isolated from blood, leukapheresis, or buffy coats obtained from healthy volunteers. Use of blood from healthy donors requires Institutional approval and oversight and appropriately trained personnel. Frozen PBMC vials collected from healthy donors by leukapheresis and processed by density gradient centrifugation are available from commercial vendors (immunospot.com, lonza.com, astartebio.com, seracarecatalog.com to name a few). Alternately, leukopacks or buffy coats are available that can be further processed using Ficoll-Hypaque density gradient (density 1.077 g/ml) centrifugation to obtain PBMCs (innov-research.com or blood banks).
3. All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless specified.
4. A corresponding AIM V media is available for clinical grade human DC generation (catalog number 087-0112DK, Invitrogen). AIM V® medium CTS™ is an FDA 510(k) cleared device which is intended for human ex vivo tissue and cell culture.
5. mMESSAGE mMACHINE® T7 Ultra kit from Ambion includes a cap analog called ARCA (23). Use of ARCA facilitates the synthesis of RNA that is capped exclusively in the correct orientation thus ensuring that all the capped RNAs are 100% functional.
6. Single-use, disposable, sterile cuvettes are available through multiple vendors including BTX online.
7. The poly(A) tail reaction adds additional ≥150 base poly(A) tail to the RNA transcripts. The poly(A) tail reaction reagents should be assembled just before use.
8. A260–A280 ratio should be greater than 1.8. The routine yield is 20–40 μg of mRNA per μg of DNA used in the in vitro transcription reaction.
9. Native gel electrophoresis in combination with A260 to A280 ratio is sufficient to judge the integrity and overall quality of mRNA. In vitro transcribed (IVT) mRNA in RNA loading

buffer, heated for 5 min at 65°C and run on a native agarose gel should show a single band of the expected size. If the in vitro transcribed mRNA produces a smear, more than one band or a band of a size other than the expected size the quality of RNA transcripts is unacceptable. This could be because the DNA template is contaminated with RNase or the transcription reaction may be prematurely terminated.

Another procedure used to determine the quality of mRNA is denaturing gel electrophoresis (24). Native (non-denaturing) gel electrophoresis is safer because it does not use hazardous chemicals, but accurate sizing of RNA molecules is not always possible under non-denaturing conditions. This is because RNA molecules have secondary structure that may affect how they migrate in a native (non-denaturing) gel. Denaturing gel electrophoresis allows an accurate determination of the size and integrity of RNA molecules.

10. For 1×10^8 PBMCs use 15 ml of AIM-V medium and a T-75 tissue culture flask.
11. The CD14+ monocytes will adhere to the tissue culture flasks during the 1–2 h incubation. T and B lymphocytes will remain non-adherent.
12. The non-adherent cells (NA), which are primarily T and B lymphocytes can be harvested and frozen for use in T cell stimulation assays with autologous DCs as stimulators. The non-adherent cells can be frozen at 5×10^7 cells/ml, 2 ml per cryovial in 90% autologous plasma (if available) or FCS and 10% DMSO and stored in a liquid nitrogen freezer.
13. DCs generally grow as loosely adherent clusters and cells that are adherent to tissue culture flasks. The loosely adherent clusters can be collected by firmly tapping the flask. The adherent DCs are harvested by incubation with cellstripper followed by vigorous pipetting. Occasionally there are a few cells stuck to the flask even after the cellstripper treatment: we usually discard those cells.
14. Supernatant saved from tube A is used to make conditioned media for DCs post-electroporation. It is therefore necessary to maintain the supernatant harvested from immature DCs in one tube (tube A) and all the DCs harvested after incubation with cellstripper in a separate tube (tube B). Conditioned media is a combination of media (supernatant from tube A) that was saved after the immature DCs were harvested plus fresh DC generation media (AIM-V) at a ratio of 1:1. Add fresh GM-CSF and IL-4 and required maturation reagents to this conditioned media. Use of conditioned media is optional.
15. Immature DC recovery should be 5–12% of the initial PBMC count with a purity of 90% or above in the final population

(by morphology and phenotype). The wide variation in cell yield is due to donor-to-donor variation. DCs can be frozen at 5×10^6 cells/ml, 2 ml per cryovial in 90% autologous plasma (if available) or FCS and 10% DMSO and stored in a liquid nitrogen freezer.

16. This is a very high cell concentration and is *critical* to achieve high transfection efficiency. Please be cognizant of the volume that is generated by the cell pellet itself and take that into account when resuspending the DCs. If in doubt, add less Opti-MEM, measure the total volume and then adjust to the required volume by adding additional Opti-MEM to achieve the desired cell concentration.
17. We use commercially available, individually wrapped, single-use, sterile disposable cuvettes and plastic transfer pipettes for electroporation.
18. Antigen encoding mRNA is used at 4 μg/10^6 DCs. RNA encoding immunostimulatory molecules is used at 10 μg/10^6 DCs. For antibody-encoding mRNA transfections, 10^6 cells are electroporated with 10 μg of heavy (H) chain antibody mRNA and 5 μg of light (L) chain antibody mRNA.
19. Air bubbles should not get trapped in the cell RNA mixture, as it will interfere with the electrical current. If you see air bubbles, you can try to eliminate them by gently tapping the cuvette.
20. If the leads are loose or the lid is not completely shut the read out on the electroporator will display that a current has passed through the cuvette, even though no current has passed through.
21. It is important to work swiftly yet gently with the cells because they are fragile after electroporation. We transfer electroporated DCs directly into DC maturation medium containing cytokines. We have not observed dead cells when the cells are harvested after 8–18 h. The recovery of DCs post-electroporation and 18 h post-maturation is between 50 and 70% of the starting DC number. If cell death does become a concern, cytokines can be added after 30 min.
22. The electroporation cuvette size is based on the number of cells that will be transfected and the maximum volume that can be used in the cuvette. We do not exceed 300 μl in a 2 mm cuvette. The square wave electroporator we use allows us to modify the voltage and pulse duration. These parameters can be modified to get maximum transfection efficiency with minimum toxicity. Square wave electroporators deliver a constant charge to the cells for a specified time. Other parameters that can be adjusted include the concentration of RNA and the buffer used for electroporation.

23. Modification to electroporation protocol for use with the 4 mm BTX cuvette.
    (a) DC concentration can be increased to 35–45 × 10^6 DCs per ml
    (b) Volume of cells per cuvette: 400–700 μl
    (c) Electroporation conditions: 500 V and 500–1,000 μs (microseconds)
24. FACS is routinely used to measure protein expression in cells transfected with mRNA. One option is to measure surface protein expression by using fluorochrome conjugated antibodies that target the protein. Another popular and convenient option is using a reporter gene, such as GFP encoding mRNA. Cells expressing GFP will fluoresce and there is no additional manipulation of the cells required. The cells are washed, resuspended in FACS buffer and analyzed for GFP expression. This is illustrated in Fig. 2b, DCs transfected with GFP mRNA are analyzed for GFP expression using FACS. In Fig. 2c, d we measure transfection efficiency by measuring the protein secreted in the supernatant of the RNA-transfected DCs.

    DCs and macrophages express Fc-receptors that may bind monoclonal antibodies nonspecifically via the Fc portion of the antibodies. Therefore, when analyzing DCs for surface protein expression, it is advisable to block these Fc receptors to prevent nonspecific antibody binding and reduce background staining on cells. This can be achieved by pre-incubating the cells with Fc-block (Human Fc Receptor Binding Inhibitor, eBioscience) for 30 min at 4°C. There is no need to wash the cells after treatment with Fc block.
25. Occasionally FACS buffers include 0.05–0.1% sodium azide. Sodium azide assists in preventing capping and shedding or internalization of the antibody–antigen complex. Staining with cold reagents/solutions and at 4°C is ideal, as low temperature prevent the modulation and internalization of surface antigens, which can produce a loss of fluorescence intensity. Sodium azide (or fixative) should not be used if the cells will be cultured for any functional analysis after FACS analysis.
26. Cells are usually stained in polystyrene round bottom 12 × 75 mm Falcon tubes. However, they can be stained in test tubes, eppendorf tubes and 96 well round-bottom plates.
27. This is usually achieved by titrating each antibody to get consistent staining without high background. For DCs we use 0.5–1 μg of antibody per 10^5 cells.
28. For 2-color staining, add all the fluorochrome-conjugated antibodies at the same time. For two- (or more-) color analysis, compensation controls must be set-up to adjust for spectral

overlap. These consist of cells stained with each fluorescent reagent separately and an additional sample containing cells stained with both colors. If two-color analysis is performed with fluorescein isothiocyanate (FITC) and phycoerythrin (PE) then controls include cells stained with FITC alone and PE alone and in addition cells stained with both FITC and PE antibodies that target a protein that is definitively expressed on the cells being used.

29. For best results, analyze the cells on the flow cytometer as soon as possible preferably on the same day. Fixing cells with 1% formaldehyde will prevent the fluorescent signal from fading.
30. Two controls should be included in every experiment: (a) Unstained control (no stain whatsoever) and (b) Isotype control (an antibody of the same isotype as the test antibody that is labeled with the same fluorochrome as the test antibody).

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Chapter 9

Large Volume Flow Electroporation of mRNA: Clinical Scale Process

Linhong Li, Cornell Allen, Rama Shivakumar, and Madhusudan V. Peshwa

Abstract

Genetic modification for enhancing cellular function has been continuously pursued for fighting diseases. Messenger RNA (mRNA) transfection is found to be a promising solution in modifying hematopoietic and immune cells for therapeutic purpose. We have developed a flow electroporation-based system for large volume electroporation of cells with various molecules, including mRNA. This allows robust and scalable mRNA transfection of primary cells of different origin. Here we describe transfection of chimeric antigen receptor (CAR) mRNA into NK cells to modulate the ability of NK cells to target tumor cells. High levels of CAR expression in NK cells can be maintained for 3–7 days post transfection. CD19-specific CAR mRNA transfected NK cells demonstrate targeted lysis of CD19-expressing tumor cells OP-1, primary B-CLL tumor cells, and autologous CD19+ B cells in in vitro assays with enhanced potency: >80% lysis at effector–target ratio of 1:1. This allows current good manufacturing practices (cGMP) and regulatory compliant manufacture of CAR mRNA transfected NK cells for clinical delivery.

Key words: mRNA transfection, NK cells, Flow electroporation, Large volume transfection, Scalability, Chimeric antigen receptor

1. Introduction

Nucleic acid transfection and transduction have been used in wide spectrum of areas including agriculture, biomedical science, and immunotherapy. Currently, most genetic modification is performed by viral vectors, which comprise high efficiency of gene transfer and low toxicity for the host cells. However, the use of viral vectors presents obvious concerns, such as random integration of viral genes in host genomes (1) and the immunogenicity of viral proteins (2). Therefore, a nonviral approach might be preferable in

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various applications. A number of nonviral DNA-transfection methods have been developed (3–5), and used in clinical trials (6–13).

Electroporation has gained popularity as one of the nonviral transfection methods. It involves the application of an electric field for loading various exogenous molecules into cells. When macromolecules, such as DNA or mRNA, are present in the cell suspension during electroporation, electrotransfection occurs. The high efficiency of nucleic acid loading, simplicity of operation and non-reliance on chemical or biological reagents make electroporation a good candidate for enhancing cellular function for therapeutic purpose. A majority of cell types tested could tolerate the electroporation process with minimal impact on viability. However, the efficiency of DNA electroporation for certain cell types, particularly hematopoietic cells which are also resistant to other nonviral transfection methods, are low (8, 14–20).

mRNA electroporation allows efficient transfer of genetic material in all cell types tested, including hematopoietic cells (21–25). mRNA electroporation transfers mRNA into the cytoplasm directly, thereby avoiding the requirement for nuclear translocation associated with DNA transfection and bypassing the endosome capture and release steps associated with other transfection methods, such as cationic liposomes or polymers. It offers benefit in providing rapid and homogeneous expression of mRNA transgenes, thereby saving time and effort, which are critical parameters in a current good manufacturing practices (cGMP) environment.

mRNA transfection also provides clear advantages over other gene transfer methods when genomic integration of transgenes is not required or even is desired to be avoided. Such applications include reprogramming somatic-cells to produce differentiating cells for therapeutic purposes or induced pluripotent stem cells (iPS) (26, 27). The requirement for sustained expression over several days for efficient cell reprogramming theoretically could be met through multiple electroporations with mRNA or protein. Thus, cells generated via mRNA transfection eliminate many of the safety concerns associated with other transfection methods, and are suited for clinical applications.

High viability and rapid transgene expression following mRNA electroporation provide potential for many therapeutic applications. It allows transfected cells to be used directly without further expansion, enabling on-site transfusional therapy. In this strategy, cells could be withdrawn from patients, processed and transferred back into patients a few hours after transfection. Remaining cells could be frozen for further dosing regimens. The early applications of mRNA transfection in therapeutics are the antigen and/or costimulatory molecule expression in DC and other antigen presenting cells (25, 28). Because of the nature of vaccine approach, large number of cell transfection and long-term antigen expression

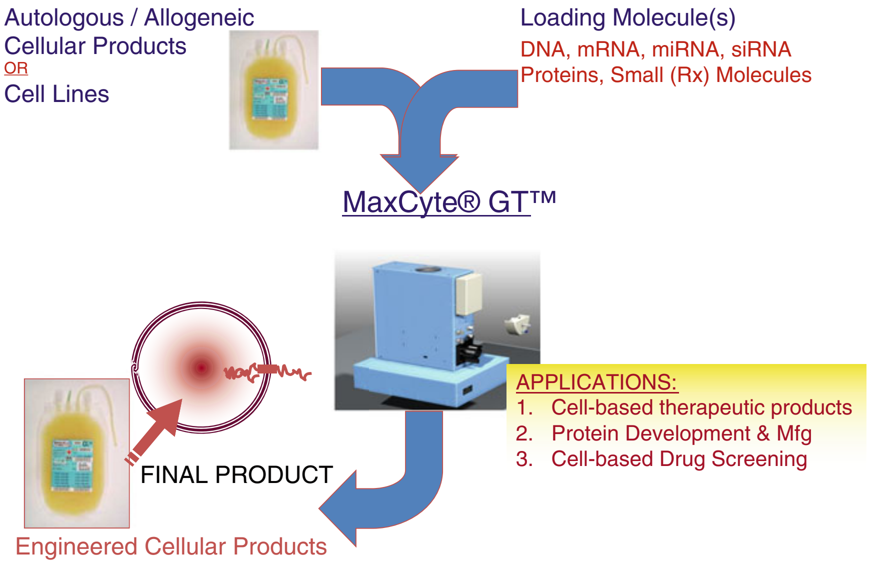
Autologous / Allogeneic
Cellular Products
OR
Cell Lines
Loading Molecule(s)
DNA, mRNA, miRNA, siRNA
Proteins, Small (Rx) Molecules
MaxCyte® GT™
APPLICATIONS:
1. Cell-based therapeutic products
2. Protein Development & Mfg
3. Cell-based Drug Screening
FINAL PRODUCT
Engineered Cellular Products

Fig. 1. Schematic for Cell processing with MaxCyte® GT™ system. Autologous/allogeneic cellular products *or* cell lines could be processed to load with DNA, mRNA, miRNA, siRNA proteins, small (Rx) molecules. The processed cells could be cell-based therapeutic products, protein development/manufacture and cell-based drug screening, either used in fresh or cryopreservation form. The whole processing time can be a few minutes for up to 1–3 × 10^{10} cells.

is not necessary (29). Recently, following the successful approach using virus infection and DNA transfection (30–35), the adoptive therapy using chimeric antigen receptor (CAR) expressed T and NK cells through mRNA transfection demonstrated therapeutic potential (23, 24). Single mRNA transfection of CAR could express the CAR and maintain targeted cell killing for 3–7 days (22) in vitro and significantly reduce tumor burden in vivo (21). However, for such therapeutic applications, large volume of mRNA transfection with a closed cGMP-compliant system is a desired and critical requirement, which can provide multiple dosing to practically prolong the effect of target cell killing, therefore compensating for the inherent transient expression feature in mRNA transfection.

Regular available electroporation systems are designed for small scale use. They employ cuvettes that allow ≤1 ml electroporation of cells from hundreds of thousands to a few dozens of million cells. They do not meet the requirements for scalability of processing large number (dozens of billions) of cells with high viability and transfection efficiency and often do not allow GMP-compliant transfection. MaxCyte® GT™ Flow Transfection System (MaxCyte, Inc., Gaithersburg, MD) (Fig. 1) fills this gap. It uses flow transfection with "closed cell processing system," nontoxic electrodes and ionic-balanced electroporation buffer. This permits automated, aseptic electroporation in highly controlled conditions with high viability & efficiency. The system showed consistent productivity from run-to-run, scalability across a wide range of scale—from R&D to clinical volumes (5×10^5 cells to 2×10^{10} cells and beyond). The system has been certified and controlled by ISO9000 and CE mark, and is regulatory supported by Master File # BBMF10702, on record with Center for Biologics Evaluation and Research (CBER), Food and Drug administration (FDA) and Health Canada. It can electroporate $1–3 \times 10^{10}$ cells in ~8 min, allows scaling up linearly of processed cells from a fraction of millions to a few dozens of billions and can be used for mRNA transfection, protein production, and virus production (22, 36, 37). It is also validated in multiple instances of development and delivery of "enhanced" potency therapeutics, including an autologous cell therapy product for treatment of cancer and multiple immune cell and stem cell therapies in clinical development in USA and Canada for treatment of immune diseases and regenerative medicine applications.

This protocol describes how to use this system to perform robust, scalable, cGMP-compliant mRNA transfections of human NK cells. NK cells and OP-1 $CD19^+$ human B cells were generously provided by St Jude Children's Hospital.

2. Materials

2.1. Reagents

1. NK medium with IL2: RPMI-1640, 10% FBS, 2 mM glutamine, 100 U/ml human recombinant IL2, antibiotics.
2. RPMI-1640 full medium: RPMI-1640, 10% FBS, 2 mM glutamine, antibiotics.
3. Cell culture flasks.
4. Cell culture plates.
5. DMSO.
6. Anti-CD56 antibody (BD Bioscience, cat # 556647).
7. Anti-CD3 (BD Bioscience cat # 554832) antibody.

2.2. Fresh NK Cells (St Jude Children's Hospital)

1. Culture NK cells in NK medium with IL2 at 5×10^5 cells/ml.
2. Pass cells to 5×10^5 cells/ml by dilution with fresh medium when cells reach to $1–2 \times 10^6$ cells/ml.

2.3. OP-1: $CD19^+$ Human B-Lineage Acute Lymphoblastic Leukemia Cell Line, Target Cells for Killing Assay (St Jude Children's Hospital)

1. Culture OP-1 cells in RPMI-1640 full medium at $3–5 \times 10^5$ cells/ml.
2. Pass cells to $3–5 \times 105$/ml when cells reach $1–2 \times 10^6$ cells/ml.

2.4. mRNA Production

1. pVAX1-based plasmid vector encoding anti-CD19 chimeric antigen receptor (CAR) (22).
2. XbaI endonuclease.
3. DNA purification kit (MinElute PCR purification Kit, cat. #28006) (Qiagen).
4. mMESSAGE mMACHINE T7 Ultra kit (cat number AM1345) (Ambion).
5. 0.5 M EDTA, pH 8.0.
6. Agarose.
7. RNase-free tips.
8. RNase-free Eppendorf tube.
9. Heating block.
10. Molecular Grade Ethanol.

2.5. mRNA Electroporation

1. MaxCyte® GT™ Flow Transfection System MaxCyte, Inc., Gaithersburg, MD (Fig. 2).
2. Small volume processing assembly (Small PA).
3. Large volume, single-use, disposable flow unit processing assembly (Large PA) (MaxCyte, Inc., Gaithersburg, MD).
4. Electroporation Buffer (MaxCyte, Inc., Gaithersburg, MD) (see Note 1).
5. Sterile 5 cc syringe.
6. Sterile 10 cc syringe.
7. Sterile 60 cc syringe.
8. 16 gauge 11/2″ needle.
9. 250 ml centrifuge bottle.
10. Sterile Eppendorf tube (1.5 ml).
11. Trypan blue solution (4%).
12. RNase-free (either human or bovine) albumin (optional).

2.6. CAR Expression Detection and In Vitro Target Cell Killing Assay

1. FACSCalibur and Cell Quest software.
2. Goat anti-mouse $(\mathrm{Fab})_2$ polyclonal antibody conjugated with biotin (cat# 115-065-072), and control antibody of goat anti-mouse Fc portion of IgG heavy chain conjugated with biotin (115-065-071) (Jackson Immuno Research Labs, West Grove, PA).
3. Peridinin chlorophyll protein-(PerCp) labeled with streptavidin (SA-PerCp) (cat#340130) (Becton Dickinson, San Jose, CA).
4. Acetoxymethyl-calcein (calcein-AM) (cat# C1430) (Molecular Probes, Eugene, OR).

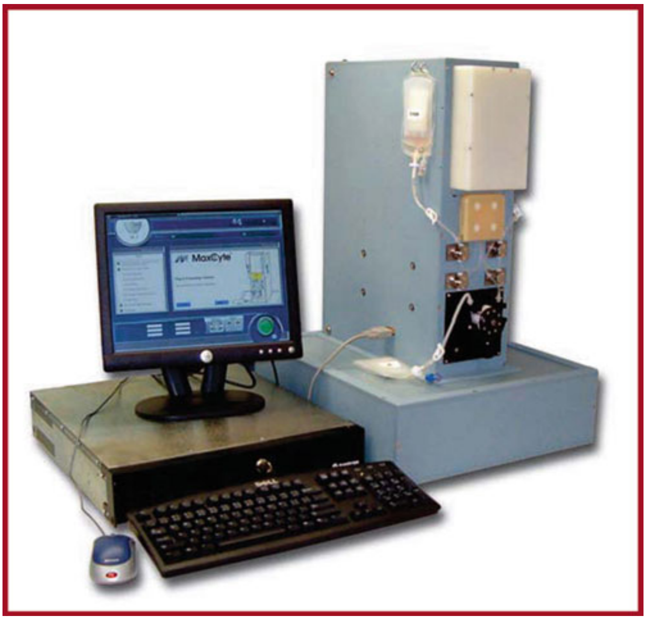

Fig. 2. MaxCyte® GT™ system. The instrument can be used in both static or flow mode. In flow mode (5–100 ml and beyond), the sample is controlled through computer-regulated peristaltic pump/valves/tube in a closed system and pumped through processing assembly for electroporation to collection bag. MaxCyte's proprietary platform technologies, certified by ISO and CE Mark, have been described in a Master File with CBER, US FDA and cross-referenced in multiple clinical trials. It provides quick, robust, scalable, cGMP-compliant transfections with high viability and efficiency (22, 36, 37).

3. Methods

3.1. mRNA Production

1. Linearize plasmid template pVAX1-CAR with Xbal.
2. Purify the linearized DNA with DNA purification kit.
3. Resuspend linearized DNA (0.5–1 mg/ml) into EB buffer (10 mM Tris–Cl, pH8.5; DNA purification kit)
4. Produce polyadenylated mRNA by using mMESSAGE mMACHINE T7 Ultra kit with using 1 μl DNase/20 μl reaction for 10–15 min at 37°C just before poly A tailing assembling (see Note 2).
5. Resuspend mRNA (1–2 mg/ml) into distill water with 0.1 mM EDTA made from 0.5 M stock EDTA.
6. Freeze mRNA at –80°C.

3.2. mRNA Electroporation of NK Cells in Small Volume (5×10^6–9×10^8)

This protocol allows electroporation with volume range 100–3,000 μl.

All operations should be carried out at room temperature.

1. Centrifuge cells at 200 × *g* for 5–10 min.
2. Aspirate the supernatant.
3. Resuspend cells in MaxCyte electroporation buffer (~10× to 20× volume of cell pellet), count the cells.
4. Centrifuge cells at 200 × *g* for 5–10 min.
5. Aspirate the supernatant.
6. Add MaxCyte electroporation buffer to obtain 5×10^7–3×10^8cells/ml (see Note 3).
7. Add mRNA to final concentration 25–300 μg/ml (see Note 4).
8. Transfer cell-mRNA mixture to Small PA.
9. Attach the Small PA to MaxCyte GT system.
10. Select preprogrammed protocol "small NK-CAR" and process the cells (see Note 5).
11. After electroporation incubate the transfected cells in the electroporation buffer at 37°C environment for 20 min to recuperate cell membrane integrity.
12. Transfer the incubated cells into pre-warmed full medium and adjust cell concentration to 5×10^5–1×10^6 cells/ml for further incubation.
13. Analyze the efficiency of transfection as it is described in Subheading 3.5.

3.3. Large Volume Electroporation of mRNA

This protocol allows electroporation with volume range 5–100 ml.

All operations should be carried out at room temperature

1. Centrifuge cells at 200 × *g* for 5–10 min.
2. Aspirate the supernatant.
3. Resuspend the cells in MaxCyte electroporation buffer (~10×–20× volume of cell pellet), count the cells.
4. Centrifuge cells at 200 × *g* for 5–10 min.
5. Aspirate the supernatant.
6. Add MaxCyte electroporation buffer to obtain 5×10^7–3×10^8/cells/ml (see Note 3).
7. Add mRNA to final concentration 25–300 μg/ml (see Note 4).
8. Transfer cell-mRNA mixture to Large PA.
9. Attach the Large PA to MaxCyte GT system.
10. Select preprogrammed protocol "NK-CAR" (see Note 5).

11. Incubate the transfected cells in the electroporation buffer at 37°C for 20 min to recuperate cell membrane integrity.
12. Transfer the incubated cells into pre-warmed full medium and adjust cell concentration to 5×10^5–1×10^6 cells/ml for further incubation.
13. Analyze the efficiency of transfection as it is described in Subheading 3.4.

3.4. Detection of the CAR Expression

1. Add 1 µl (1.5 mg/ml) anti-$(\mathrm{Fab})_2$ or control antibody (anti-Fc portion of IgG heavy chain) to 100 µl cells (1-4e5 cells) in full culture medium.
2. Incubate at 4°C for 20 min.
3. Prepare washing medium (PBS + 5% RPMI full medium) and place it in ice.
4. Add 3 ml washing medium to the stained cells in 5 ml-FACS tube.
5. Centrifuge the cells at 400 × *g* for 5 min at room temperature.
6. Decant the washing medium.
7. Repeat steps 4–6 one more time.
8. After decant the washing medium, add 3 µl SA-PerCp.
9. Stain cells for 5–20 min in ice.
10. Repeat steps 4–6.
11. Add 0.25–0.4 ml washing medium.
12. Analyze cells by flow cytometry.

3.5. Target Cell Killing Assay

1. Take 10 ml of growing target OP-1 cells.
2. Add calcein-AM to the target cells at final concentration of 60 nM.
3. Invert the tube 2–3 times to make calcein-AM be suspended uniformly.
4. Incubate for 0.5-1 min at room temperature.
5. Wash three times with 10 ml of full medium by centrifugation (200 × *g*/5 min) at room temperature.
6. Resuspend cells in RPMI-1640 full medium at 10^6 cells/ml (see Note 6).
7. Transfer 100 µl of cell suspension (10^5 cells) to 96-well U-bottom tissue culture plates in replicates.
8. Add 100 µl of either full medium alone (control) or non-transfected or transfected effector cells in full medium with different concentrations to the well, to achieve various effector–target [E/T] ratios.

9. Centrifuge the plate with $400 \times g \times 5\text{min}$ at room temperature.
10. Culture the cells with desired culture time for cell killing in 37°C culture incubator (see Note 7).

3.6. Analysis of Cell Killing

1. Resuspend cells in the 96 well by pipeting
2. Transfer cells from the well into FACS tube
3. Determine cell killing rate by flow cytometry with 20–30s timely controlled FACS collection mode (see Note 8)

   Calculate cell lysis in the following formula:

$$100 - \frac{N_{\text{target}}}{N_{\text{target}-\text{control}}} \times 100,$$

where N_{target} is the number of viable target cells when mixed with effector cells and the $N_{\text{target-control}}$ is the same number of target cells cultured in the medium alone.

4. Notes

1. 0.5% of RNAse-free albumin could be added to electroporation buffer. It may increase cell viability.
2. Instruction with mMESSAGE mMACHINE T Ultra 7 kit (Ambion) describes protocol to make 20 μl volume for mRNA production in one reaction tube and purify by LiCl precipitation. If one needs to produce more mRNA, one could increase the reaction volume to up to 100 μl (5 reactions) following the same instruction using the same 1.5 ml eppendorf tube, with proportionally increased other reagents. If one desire to produce even more mRNA, one could increase the volume to be 200 μl (10c reactions), but use 15 ml centrifuge tube for mRNA production.
3. Cell density in this range results in similar transfection efficiency.
4. The transfection efficiency will be in proportional to the amount of mRNA used, but the viability will not change in the range of mRNA amount listed.
5. MaxCyte GT system provides both small and large volume transfection modes. Training and technique support is provided for PA and system use. Detailed operation manual and general protocols are also provided. In general, pilot transfection with small volume is usually performed before large volume transfection for time and cost saving. However, to achieve maximal efficiency and viability for any particular cell type,

especially primary cells, the transfection condition might be needed to be optimized. The optimal transfection condition found in small volume transfection with MaxCyte GT system can be directly transferred to large volume transfection (up to 100 ml with 1–3 × 10^{10} cells). The processing time of flow electroporation is proportional to the processing volume. This runs for approximately 6–10 min to transfect 1–3 × 10^{10} cells. If needed, special transfection protocols with special arrangement could be developed and added to the system with assistance from Maxcyte support team.

6. If target cell number is limited, a frequently encountered situation when using primary tumor cells as the target cells, reduced target cell concentration can be used.
7. Cell killing can be observed 3–24 h after mixing. One can choose a mixing duration for killing study.
8. Perform the FACS analysis with time collection mode for measuring target cell killing. Two separated populations of cells will be shown in FACS analysis, the unlabeled effector NK cells and the calcein-labeled target cells (e.g., OP-1) in green fluorescence channel. Identify the viable and labeled target cells and set up gating using labeled control target cells. When some target cells are killed, the killed target cells will have lower fluorescence intensity, and move out of the region of the gating. Record the viable target cell number. The decrease of the number of the viable target cells from the number of control target cells indicates the killing of target cells. When fewer target cells are used, the time for the FACS collection needs to be increased accordingly.

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Chapter 10

Retrovirus-Based mRNA Transfer for Transient Cell Manipulation

Melanie Galla, Axel Schambach, and Christopher Baum

Abstract

Retrovirus-mediated mRNA transfer (RMT) combines the advantageous features of retrovirus-mediated cell targeting and entry with the controlled transfer of mRNAs. We have recently exploited this strategy for the dose-controlled transfer of recombinases and DNA transposases, avoiding cytotoxicity and potential insertional mutagenesis. Further applications can be envisaged, especially when low expression levels are sufficient to modify cell fate or function. Here we describe a step-by-step protocol for the generation of RMT vector particles, their titration and their application in a model cell line.

Key words: RMT, mRNA transfer, Gammaretroviral vector, Murine leukemia virus, Transient, Dose-controlled, Non-cytotoxic, Cell targeting

1. Introduction

Transient and dose-controlled cell modification is increasingly interesting in cases where a potentially toxic or a temporally regulated protein needs to be delivered. However, some currently existing transient delivery methods, e.g., physicochemical transfection and retroviral transfer of episomes, still suffer from high cytotoxicity, low efficiency and/or residual integration events. Therefore, the development of new transient expression methods is highly desirable.

Recently, we have developed retrovirus-mediated mRNA transfer (RMT), a new strategy for the delivery of mRNA molecules into target cells. RMT is a nonreplicating, Murine Leukemia Virus (MLV)-based, gammaretroviral vector platform that allows the transient and nontoxic expression of foreign proteins in target cells. We could show that RMT depends on retroviral particle formation,

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the presence of a viral glycoprotein as well as the encapsidation of the viral mRNA genome (1–3) (see also below).

Retroviruses are RNA viruses, which are evolutionarily optimized to penetrate susceptible cells in a receptor-mediated manner. The host tropism is determined by the viral glycoproteins, which are deposited in a lipid envelope derived from the membrane of the previous host cell. The genome of retroviruses comprises two identical copies of a linear, plus- and single-stranded RNA molecule, which are embedded within the viral capsid (Fig. 1). Encapsidation of the retroviral genome occurs during particle assembly and is strictly dependent on the retroviral packaging signal (Ψ), a highly structured RNA motif located upstream of the viral coding sequence (4). After cellular entry and a poorly understood uncoating step, retroviruses reverse transcribe their genome into one copy of double-stranded DNA, which is then subsequently integrated into the host cell's genome (5). Reverse transcription is initiated by a cellular tRNA molecule, which is packaged during viral assembly and binds to a specific 18 bp-long sequence on the viral RNA genome, the primer binding site.

Since each of the two viral genomic RNA copies contains a CAP structure and a polyA tail, and thus resembles a cellular mRNA, we speculated that preventing retroviral vector particles from reverse transcription might result in mRNA delivery vehicles that introduce any type of mRNA into a given target cell. Once cellular entry has occurred, these particles would release their nucleic cargo into the cytoplasm, where the engineered vector mRNAs could then serve as direct translation templates for any protein of interest (instead of being reverse transcribed). There are multiple possibilities to interrupt reverse transcription. It can be blocked by the following:

(A) Nucleoside analogs, such as 3′-azido-3′deoxythymidine (AZT), which block reverse transcription through the incorporation of false nucleosides; (B) direct mutations in the catalytic domain of the viral reverse transcriptase; and (C) mutations in the PBS sequence, thereby inhibiting tRNA primer binding and consequently, reverse transcription.

Since the bound tRNA primer may potentially interfere with ribosomal scanning during CAP-dependent translation, we focussed on the last approach and introduced an artificial primer binding site (aPBS), designed by Pedersen and colleagues not to match any naturally occurring tRNA molecule (6), into our MLV-based gammaretroviral vectors (Fig. 1). This approach abrogated reverse transcription efficiently, i.e., >1,000-fold, and resulted in better transgene expression when compared to approaches where wild-type vector particles were blocked by AZT (1). This supports the hypothesis that a bound tRNA primer might interfere with ribosomal scanning and therefore efficient translation.

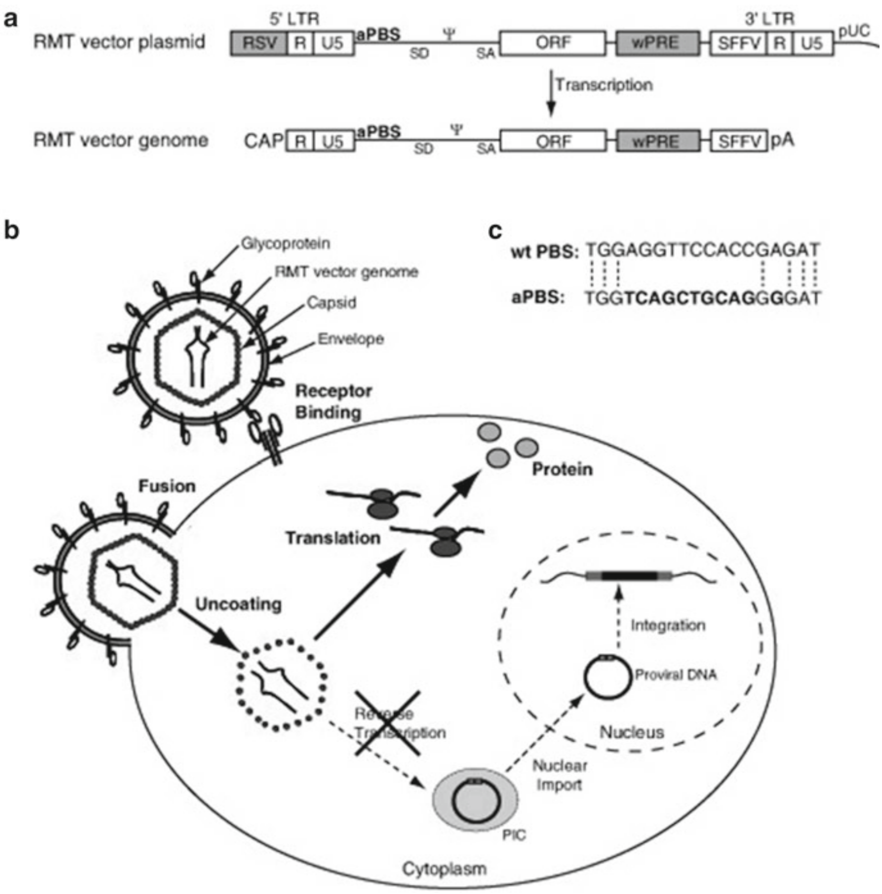

Fig. 1. Retrovirus mediated mRNA transfer (RMT) for transient cell modification. (**a**) The RMT vector plasmid (pRSF91aPBS. ORF.PRE) was optimized to improve the level of vector mRNA genomes in producer cells. The MLV-based RMT vector is embedded in a pUC plasmid backbone and contains the two long terminal repeats (5′LTR and 3′LTR) that flank the artificial primer binding site (aPBS), a splice donor (SD), a splice acceptor (SA), the viral packaging signal (Ψ) and the transgene cassette with the open reading frame of interest (ORF). The introduction of rather strong enhancer/promoter sequences from Rous Sarcoma Virus (RSV) results in high transcript levels of RMT vector genomes (3), which resemble cellular mRNAs with their CAP-structure and their polyA tails. The posttranscriptional regulatory 900 bp-element from Woodchuck Hepatitis Virus (wPRE) enhances polyadenylation and vector mRNA stability. (**b**) RMT vector particles contain two plus-stranded RNA vector genomes, which are embedded in the capsid core. After receptor-mediated entry of RMT particles, uncoating takes place and releases the mRNA genomes into the cytoplasm for immediate translation of the protein of interest (*see black solid arrows*). Due to inhibition of reverse transcription, all subsequent steps of the retroviral life cycle are blocked (nuclear import, integration; *see dashed arrows*). (**c**) In the artificial primer binding site (aPBS) of RMT vectors, 11 out of 18 nucleotides are mutated when compared to a wild-type vector encoding the primer binding site of murine embryonic stem cell virus (wt PBS; (47)). This prevents binding of naturally occurring tRNA molecules, thus blocking initiation of reverse transcription.

We chose MLV-based vectors because of their relatively short 5′ untranslated region and eliminated residual AUG codons hampering recognition of the AUG of the protein coding sequence (7). This would have been even more difficult for lentiviral vectors having a more complex leader sequence with various potential ORFs. In addition to the aPBS sequence and improved leader region, we further optimized our RMT vectors to improve transgene expression

(Fig. 1a): (a) where possible, we codon-optimized the transgene of interest improving mRNA stability and translatability (see Note 1); (b) we enhanced the generation of vector mRNA in producer cells by incorporating strong enhancer/promoter sequences (8); and (c) we introduced the posttranscriptional regulatory 900 bp-element of the Woodchuck Hepatitis Virus (wPRE) to improve polyadenylation and thus mRNA stability (see Note 2). We chose the most potent variant available based on a screen of various wPRE sequences (9–11) (and data not published). The use of RMT vectors for RNA transfer does not lead to transgene integration into host genomes; this reduces the potential safety concerns associated with the presence of the X-protein open reading frame in this hepadnaviral sequence (12).

Like wild-type MLV vectors, RMT particles can be easily packaged using a so-called split-packaging design (13), which involves co-transfecting the RMT vector (pRSF91aPBS.ORF.PRE) together with MLV gag/pol and envelope/glycoprotein helper plasmids. Separating the vector from coding sequences of gag/pol and envelope/glycoprotein reduces the risk of replication-competent virus generation. It also allows pseudotyping of the particles with a variety of different envelopes/glycoproteins (for a selection see Table 1) to control specificity of cell targeting. We were previously able to show that retroviral particles pseudotyped with the murine ecotropic envelope/glycoprotein selectively transduce murine cells in a mixed population of human and mouse fibroblasts (1).

The split packaging design allows the introduction of important controls:

By omitting gag/pol or the envelope/glycoprotein from the transfection mix, we could demonstrate the dependence of RMT

Table 1
Scheme for the generation of RMT vector particles. Plasmid amounts for one transfection reaction on a 10 cm-culture dish are given. Alternative envelope proteins and their tropism are indicated

Vector	Gag/Pol	Envelope protein	Tropism
pRSF91aPBS.ORF.PRE 5 μg Ref. (3)	pcDNA3.MLVg/p 7 μg Ref. (16)	Ecotropic: 3 μg Ref. (23)	Rodents
		RD114-TR: 2 μg Ref. (24)	Cat, Human Macaque
		VSV-G: 2 μg Ref. (17)	Broad

ORF open reading frame, *VSV*-G glycoprotein of Vesicular Stomatitis Virus, *RD114/*TR envelope of feline endogenous retrovirus fused to the cytoplasmic tail (TR) of the amphotropic envelope protein on the formation of retroviral particles and cellular receptors (1, 3). Furthermore, particle mediated mRNA transfer was abrogated when replacing the RMT vector plasmid by a corresponding nonviral expression vector lacking Ψ, indirectly arguing that the aPBS vector genomes are the main component of RMT (1). This could be confirmed by expressing short hairpin RNAs in target cells that recognize the incoming retroviral aPBS vector genomes, thereby clearly reducing RMT-mediated transgene expression (2).

RMT is applicable for a variety of transgenes and target cells. Recently, we have used this technique for highly efficient and transient transfer of mRNAs encoding DNA-modifying enzymes, such as FLP and CRE recombinases as well as the *Sleeping Beauty* transposase (1, 3). Interestingly, in contrast to integrating retroviral vectors, RMT enabled the dose controlled and nontoxic expression of these proteins. Transduction of other mRNAs coding EGFP (enhanced green fluorescent protein) (1, 2) or membrane-located truncated version of human CD34 (unpublished data) were also successful. In our experience, RMT works best in applications in which low and transient expression of proteins achieve the desired biological effects. High overexpression mediated by RMT is unrealistic as every particle contains only two strands of genomic mRNA and the release of the mRNA for free access of ribosomes may be rate-limiting. The transient nature of the system allows expression of proteins that may have toxic effects upon continuous expression and is especially suited for cell types which do not tolerate physicochemical transfection procedures. Another potential advantage is the option to equip retroviral RMT particles with receptor-specific envelopes/glycoproteins and thus to use the technology for cell-specific mRNA targeting (1, 14).

In summary, RMT represent a useful and novel platform for mRNA delivery into target cells. Here we describe a step-by-step protocol for the generation of RMT vector particles. We provide an example of how transduction is performed using this strategy in SC-1-derived FLP reporter cells (3, 15) (Fig .2) and give a protocol for the relative quantification of RMT particles.

2. Materials

All materials to be used for cell culture work must be sterile. If not stated otherwise, all solutions should be prepared with RNase- and DNase-free water.

2.1. Cell Culture

1. Human 293T cells.
2. Human HeLa cells.
3. FLP reporter cells derived from murine SC-1 fibroblasts (3, 15).

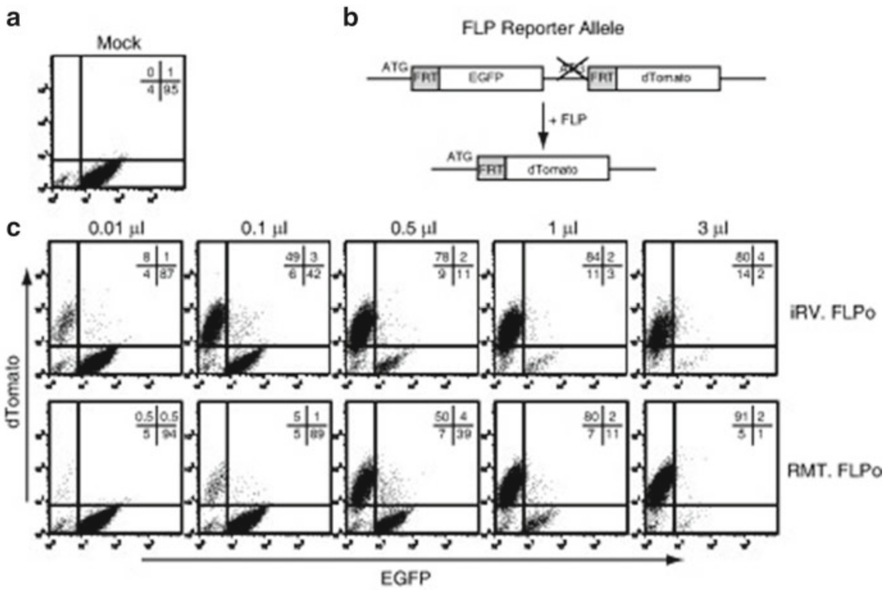

Fig. 2. Efficient transfer of FLP recombinase into murine fibroblasts via RMT. (**a**) Flow cytometry of murine FLP reporter fibroblasts (SC-1-based), which have been transduced with a lentiviral vector harboring the FLP reporter allele depicted in (**b**). Upon FLP administration, the green fluorescent protein (EGFP) cassette that is flanked by two FLP/FRT recognition sites is excised. A red fluorescent dTomato cassette, previously lacking an ATG, is subsequently activated, indicating productive FLP activity. (**c**) FLP reporter cells were transduced with serial dilutions of concentrated RMT or integrating (iRV) vectors transferring a codon-optimized version of FLP recombinase (FLPo).

4. High-glucose [4.5 g/L] Dulbecco's modified Eagles medium including stable glutamine (DMEM).
5. Heat-inactivated fetal calf serum (FCS) (see Note 3).
6. 100 mM Sodium pyruvate solution.
7. Penicillin–Streptomycin stock solution (100×).
8. Phosphate-buffered saline (PBS).
9. Trypsin/EDTA stock solution (10×).
10. T-75 cell culture flasks (see Note 4).
11. Disposable serological pipettes of different sizes (1–25 mL).
12. Electronic pipette filler.

2.2. Production of RMT Vector Particles (see Table 1)

1. Plasmids: RMT vector plasmid (3).
2. MLV gag/pol expression plasmid (16).
3. Expression plasmid encoding the envelope/glycoprotein from Vesicular Stomatitis Virus (VSV-G; (17)) (see Note 5).
4. pCMV-DsRed-Express expression plasmid (BD Biosciences Clontech, Heidelberg, Germany) (see Note 6).
5. RNase- and DNase-free ultrapure water.

6. 2× HeBS transfection solution (pH 7.05): 50 mM Hepes, 280 mM NaCl, and 1.5 mM Na_2HPO_4. Adjust with NaOH. Store at −20°C.
7. 2.5 M $CaCl_2$ solution. Store at −20°C.
8. 1,000× chloroquine (catalog number C6628, Sigma Aldrich, Munich, Germany) stock solution [25 mM]. Store at −20°C.
9. Human 293T producer cell line.
10. 1 M Hepes buffer solution (catalog number S11-001, PAA Laboratories, Coelbe, Germany).
11. Transfection medium (TFM): Complete DMEM culture medium (see Subheading 3.1) including 20 mM Hepes.
12. Surface-treated (growth area) 10 cm-culture dishes.
13. 1.5 mL Eppendorf reaction tubes.
14. 15 mL conical tubes.
15. 50 mL conical tubes.
16. Disposable serological pipettes of different sizes (1–25 mL).
17. Electronic pipette filler.
18. Disposable pipette tips.
19. Adjustable micropipettes (20 μl, 200 μl, and 1,000 μl).
20. Disposable Millex-GP syringe filter, pore size 0.22 μm (Millipore, Schwalbach/Ts., Germany).
21. Disposable 10 mL syringes.
22. Hemocytometer for counting cell numbers.

2.3. Concentration of RMT Vector Particles

1. Cell-free supernatant(s) of RMT vector particles produced as described in Subheading 3.2 (see Note 7).
2. Polyallomer centrifuge tubes (25 × 89 mm) (catalog number 326823, Beckman Coulter, Krefeld, Germany).
3. Optima LE-80K ultracentrifuge (Beckman Coulter).
4. Ultracentrifuge Rotor SW28 or SW32Ti (Beckman Coulter).
5. Analytical balance.
6. TFM for resuspension of the retroviral pellet (see Note 8).
7. 1.5 mL Eppendorf reaction tubes.
8. Disposable serological pipettes.
9. Electronic pipette filler.
10. Disposable pipette tips.
11. Adjustable micropipettes (20 μl, 200 μl and 1,000 μl).
12. Bacillol Plus solution for decontamination (Bode Chemie, Hamburg, Germany) (see Note 9).

13. Vacuum pump for thorough removal of remaining supernatant from the polyallomer tube after concentration and decantation.
14. Paper towels.

2.4. Relative Quantification of RMT Vector Particles

1. Concentrated RMT supernatant(s) that were generated as described in the Methods section (see Subheadings 3.2 and 3.3).
2. HeLa cells.
3. Complete DMEM culture medium (see Subheading 3.1).
4. PBS.
5. FACS buffer: PBS with 2% heat-inactivated FCS.
6. Surface-treated 6-well culture plates.
7. Disposable serological pipettes.
8. Electronic pipette filler.
9. Disposable pipette tips.
10. Adjustable micropipettes (20 μl, 200 μl and 1,000 μl).
11. 1,000× protamine sulfate (catalog number P4505, Sigma Aldrich) stock solution [4 mg/mL]. Store at −20°C.
12. RNase- and DNase-free water.
13. Temperature-regulating centrifuge with rotatable tissue culture plate holders (Multifuge 3S-R; Heraeus, Berlin, Germany).
14. Tabletop centrifuge for Eppendorf reaction tubes.
15. RNAzol-B solution (WAK Chemicals, Steinbach, Germany).
16. Chloroform.
17. 2-propanol.
18. 100% ethanol.
19. 75% ethanol (see Note 10).
20. RiboLock RNase inhibitor [40 U/μl] (catalog number EO0381, MBI Fermentas, St. Leon-Rot, Germany).
21. TURBO DNase [2 U/μl] (catalog number AM2238, Ambion, Kassel, Germany).
22. RNAeasy Mini Kit (catalog number 74104, Qiagen, Hilden, Germany).
23. QuantiTect Reverse Transcription Kit (catalog number 205311, Qiagen).
24. QuantiTect SYBR Green PCR Kit (catalog number 204143, Qiagen).
25. Human β-actin and wPRE primer pairs: human β-actin: 5′-CCTCCCTGGAGAAGAGCTA-3′ (Forward) and 5′-TCCATGCCCAGGAAGGAAG-3′ (Reverse) (see Note 11); wPRE: 5′-GAGGAGTTGTGGCCCGTTGT-3′ (Forward) and

5′-TGACAGGTGGTGGCAATGCC-3′ (Reverse) (all Eurofins MWG Operon, Ebersberg, Germany). Each diluted to 15 μM with water provided in the Qiagen's Quantitect SYBR Green PCR Kit.

26. MicroAmp Fast Optical 96-Well Reaction Plate (catalog number 4346906, Applied Biosystems, Darmstadt, Germany).
27. MicroAmp Optical Adhesive Film Kit (catalog number 4313663, Applied Biosystems).
28. StepOnePlus Real-Time PCR System (catalog number 4376600, Applied Biosystems).

2.5. Transduction of Target Cells with RMT Particles

In principle, RMT vector particles should allow the mRNA transfer of all kinds of proteins into given target cells. We succeeded in transferring mRNAs of DNA-modifying enzymes as well as EGFP as an example for a cytoplasmic protein (1–3). For simplicity, we will here describe the transfer of a codon-optimized FLP recombinase (FLPo; (18)) into SC-1-derived FLP reporter cells via concentrated RMT vector particles.

1. SC-1-based FLP reporter cells (3, 15).
2. Transduction medium: Complete DMEM culture medium including 4 μg/mL protamine sulfate.
3. RMT vector particles transferring FLPo mRNAs (see Note 12).
4. Surface-treated 12-well culture plates (see Note 13).
5. Conical 50 mL tubes.
6. Disposable serological pipettes.
7. Electronic pipette filler.
8. Pipette tips.
9. Adjustable micropipettes (20 μl, 200 μl and 1,000 μl).
10. Hemocytometer.
11. Temperature-regulating centrifuge with rotatable tissue culture plate holders.

3. Methods

3.1. Cell Culture

Grow human 293T and HeLa cells in complete DMEM culture medium (see below). Split confluent cultures every 2–3 days in a ratio of 1:10–1:15. Detach cells by washing once with PBS and subsequently add 1x Trypsin/EDTA for 3–5 min at 37°C with 5% CO_2. The following need to be prepared before starting culturing the cells:

1. Prepare complete DMEM culture medium supplemented with 10% (v/v) heat-inactivated FCS, 1 mM sodium pyruvate and 1% (v/v) Penicillin/Streptomycin.
2. Dilute 10× Trypsin/EDTA stock solution with PBS to obtain a 1× Trypsin/EDTA working solution.

3.2. Production of RMT Vector Particles

For the best possible experimental outcome, we recommend to concentrate all RMT vector supernatants. Following the concentration protocol that is mentioned below, 32–34 mL of RMT supernatant per polyallomer ultracentrifuge tube is optimal. We therefore always perform double transfection reactions for each RMT vector preparation. The resulting retroviral supernatants are pooled after harvesting and subsequently concentrated. For simplicity, the plasmid and reagent quantities for only one transfection reaction are listed.

1. The day before transfection, seed $5–6 \times 10^6$ 293T cells per 10 cm-culture dish in 10 mL of complete DMEM culture medium (see Note 14).
2. Twenty-four hours later, bring the plasmids and reagents necessary for transfection (RNase- and DNase-free water, 2× HeBS solution, 2.5 M $CaCl_2$ solution) to room temperature. Thaw the 1,000× chloroquine stock solution and keep it on ice.
3. Prepare the transfection medium (TFM) by adding 20 mM Hepes to complete DMEM culture medium. Prewarm in a water bath to 37°C.
4. Combine 5 μg RMT vector plasmid, 7 μg MLV gag/pol expression plasmid and adequate amounts of the desired envelope/glycoprotein expression plasmid (see Table 1) in a 1.5 mL Eppendorf tube. To control for transfection efficiencies, include 1 μg of nonviral pCMV-DsRed-Express expression plasmid per transfection (see Note 6). Dilute all plasmids with water to a total volume of 450 μl and add 50 μl of 2.5 M $CaCl_2$ solution.
5. Add 500 μl of 2× HeBS solution to a conical 15 mL tube, place a disposable 1 mL serological pipette (see Note 15) in the tube and start to blow air into the 2× HeBS solution using the electronic pipette filler. While still air bubbling with one hand, add with the other hand the DNA–$CaCl_2$ mixture dropwise to the 2× HeBS solution. Air bubble everything for another 30 s and incubate the mixture for 20 min at room temperature to allow efficient formation of DNA–calcium phosphate precipitates.
6. In the meantime, prepare adequate amounts of TFM (10 mL per transfection reaction) containing chloroquine at a final

concentration of 25 μM. By using disposable 10 mL serological pipettes and the electronic pipette filler, remove the old culture medium from seeded 293T cells and carefully add 10 mL of chloroquine-containing TFM to each 10 cm-dish (see Note 16). Do one dish after the other (see Note 17).

7. After the 20 min incubation time, add dropwise the DNA–calcium phosphate mixture from step 5 to the 293T cells. Gently rock the plates back and forth to mix the drops with the medium.
8. Incubate the cells for at least 6 h but not longer than 16 h in an incubator at 37°C with 5% CO_2 (see Note 18).
9. Remove the chloroquine-containing TFM from the cells and add 10 mL of freshly prepared TFM without chloroquine.
10. Thirty-six hours after adding the DNA–calcium phosphate precipitates to the cells, harvest the first time RMT vector particles that were produced and released into the TFM by the transfected 293T cells. Therefore, carefully draw up the medium with a 10 mL syringe, attach a Millex-GP syringe filter (0.22 μm) to the filled syringe and filter the "36 h-supernatant" into a conical 50 mL tube. Place the tube on ice and provide the cells with 10 mL of fresh TFM (without chloroquine) immediately using a 10 mL serological pipette. Return the cells to the incubator and store the supernatant in the refrigerator overnight.
11. After another 24 h incubation time, collect RMT vector particles a second time. Since this would be 60 h after adding the DNA–calcium phosphate precipitates to the cells, the harvest is termed "60 h-supernatant."
12. Combine the "36 h-supernatant" with the 10 mL of the "60 h-supernatant" (see Note 19).
13. Proceed directly with RMT particle concentration or store the combined supernatants at –80°C until further usage (see Note 20).

3.3. Concentration of RMT Vector Particles

To achieve best RMT performance, we suggest concentrating the produced viral supernatants at least ~50× via ultracentrifugation. When performing the protocol, be aware of that the half-life of retroviral particles depends on temperature. Therefore, try to keep the supernatants on ice and minimize all manipulations provided at room temperature.

1. Transfer 32–34 mL of each RMT supernatant produced as described in Subheading 3.2 into a sterile polyallomer centrifuge tube (see Note 21).
2. Precisely counterbalance all tubes and place them into sterile buckets of the rotor (see Note 22).

3. Centrifuge the RMT vector particle supernatants overnight at 13,238 × *g* and 4°C (see Note 23).
4. Decant the supernatants after centrifugation and place the polyallomer tubes upside down on a paper towel soaked with Bacillol Plus solution for draining (see Note 9).
5. Remove the remaining drops of supernatant using a vacuum pump and immediately add ice-cold TFM or other resuspension medium to the viral pellets (see Note 8).
6. Gently resuspend the pellets, aliquot into smaller portions on ice and store at −80°C until usage (see Note 24).

3.4. Relative Quantification of RMT Vector Particles

Retroviral vector supernatants need to be quantified to assess their efficacy of target cell transduction. This can either be done by measuring physico-biochemical components of the retroviral particle (e.g., viral protein load and/or the genomic mRNA content) or by analyzing the infectivity of a retroviral supernatant on given indicator cells (biological titer). Common methods to determine the viral protein load of a supernatant are retroviral capsid ELISAs or reverse transcriptase activity assays. The total amount of viral mRNA genomes within a preparation can be measured by real-time RT-PCR. Instead, the "biological titer" of a retroviral vector preparation indicates the amount of functional retroviral particles per mL supernatant. Defective (i.e., not productive in infection) or empty (i.e., particles without retroviral mRNA genomes) are—in contrast to the physical methods described above—not taken into account.

We were recently able to demonstrate that the retroviral genomic RNA content of gammaretroviral supernatants correlates better with the biological titer than the viral protein load (2). We therefore recommend performing real-time RT-PCR for particle quantification rather than methods that are based on the determination of retroviral proteins. To avoid the acquisition of defective particles containing retroviral genomic RNAs, we decided to measure the retroviral genomic RNA content within target cells shortly after entry. This also has the advantage that the retroviral genomic RNA genome can be normalized to a housekeeping mRNA (e.g., β-Actin) for comparison (see Note 25). Theoretically, a variety of cell lines can be used for particle titration (see Note 26). We here give an example for the titration of VSV-G-pseudotyped RMT particles on human HeLa cells.

1. The day before transduction, seed 7–8 × 10^5 HeLa cells per well of a 6-well-plate.
2. Twenty-four hours later, thaw one aliquot of the concentrated VSV-G-pseudotyped RMT supernatants on ice, and prewarm PBS and complete DMEM culture medium to 37°C.
3. Prepare the transduction medium consisting of warm complete DMEM culture medium with 4 μg/mL protamine sulfate (see Note 27).

4. Aspirate the used culture medium from seeded cells and add 1 mL transduction medium to each well of the 6-well-plate. Add 20–30 μl of concentrated RMT supernatant per well, pipette up and down and centrifuge the plates for 1 h at 863 × *g* and 30–37°C (so called spinoculation) (see Note 28).
5. Transfer the plates to the CO_2-incubator and incubate for 1 h.
6. Remove the viral particle containing transduction medium, wash cells twice with 2 mL of warm PBS and add 2 mL complete DMEM culture medium.
7. Incubate the cells for another hour in the CO_2-incubator, so that RMT particles that are bound to the cell surface can be internalized.
8. Remove DMEM culture medium, wash cells once with 2 mL warm PBS and add 700 μl 1× Trypsin/EDTA. Incubate the plates for 3–5 min in the CO_2-incubator. After detachment of the cells from the well's surface, add 800 μl of ice-cold FACS buffer on top to inactivate trypsin. Transfer the cell suspension into a 1.5 mL Eppendorf reaction tube and pellet the cells for 5 min at 800 × *g* and 4°C in a tabletop centrifuge. Resuspend the cells in 1 mL ice-cold PBS, centrifuge again, discard the supernatant and place the samples on ice.
9. Thoroughly homogenize the cells with 1 mL RNAzol-B. Proceed with extracting the RNA or store the homogenized cells at −20°C until further usage.
10. If stored at −20°C, thaw the frozen samples on ice, otherwise proceed with step 11.
11. Add 200 μl of chloroform to each RNAzol homogenate. Pulse-vortex each sample for at least 30 s and centrifuge for 10 min at 17,000 × *g* and 4°C.
12. Take the aqueous phase (~450 μl) and precipitate the RNA by addition of 500 μl ice-cold 2-propanol. Spin the tube for 30–45 min at 17,000 × *g* and 4°C.
13. Add 1 mL of 75% ethanol to the RNA pellets, spin at 17,000 × *g* and 4°C for 3 min and remove the ethanol with a 1 mL-adjustable micropipette.
14. Repeat the washing procedure from the previous step.
15. Centrifuge again and thoroughly remove remaining 75% ethanol with a 200 μl- and/or 20 μl-adjustable micropette.
16. Immediately dissolve RNA pellets in adequate amounts of RNase- and DNase-free water by shaking on a Thermo-shaker for 10 min at 50°C (see Note 29). To inhibit potential RNases, add 1 μl of RiboLock RNase inhibitor to each sample. Store at −20°C until further usage.

17. Thaw the RNA samples on ice. Take an aliquot of 3–5 μg RNA and dilute with water to a final volume of 25 μl. Add 3 μl 10× TURBO DNase Buffer (provided with the TURBO DNase) and 2 μl TURBO DNase [2 U/μl] to the reaction. Digest potential DNA contaminations by incubating the samples at 37°C for 1 h.
18. Remove the TURBO DNase from the RNA sample by using the "RNA clean up" protocol from the RNAeasy Mini Kit according to Qiagen's instructions (see Note **30**): In brief, shortly centrifuge the tube to remove condensed water from the inside of the lid. Adjust the sample with 70 μl of RNase-free water (supplied with the kit) to a volume of 100 μl. Add 350 μl RLT buffer (provided) and thoroughly pipette up and down. Add 250 μl 100% ethanol (not provided), mix well by pipetting and directly transfer the RNA sample to an RNeasy Mini spin column (provided). Spin the column for 15 s at 11,600 × *g* in a tabletop centrifuge. Discard the flow-through. Wash the column with 500 μl buffer RPE (provided) by spinning at 11,600 × *g* for 15 s. Discard the flow-through. Add another 500 μl RPE washing buffer and centrifuge at 11,600 × *g* for 2 min. Place the column in a new 2 mL-collection tube (provided) and spin at full speed for 1 min. Place the column in a new 1.5 mL-collection tube (provided) and add 35 μl of RNase-free water directly to the spin column membrane. Incubate for 1 min at room temperature and then elute the RNA by centrifuging for 1 min at 11,600 × *g*. Continue with step 19 or store the samples at −20°C until further usage.
19. Repeat DNase digestion and RNA clean up of the samples as described in steps 17 and 18. Continue with step 20 of the protocol or store the samples at −20°C until further usage.
20. Reverse transcribe the DNase-treated RNA samples with the QuantiTect Reverse Transcription Kit as follows (protocol supplied with the kit): Dilute ~600 ng of RNA with RNase- and DNase-free water (provided) to a total volume of 12 μl, add 2 μl of 7× gDNA Wipeout Buffer (provided) and incubate for 2 min at 42°C. Immediately place the tubes on ice, add 4 μl of 5× Quantiscript RT Buffer (provided) and 1 μl RT Primer Mix (provided) to the reaction. Deviating from the provided protocol, split the reaction (=19 μl) into two parts and add either 0.5 μl Quantiscript Reverse Transcriptase (provided) or 0.5 μl water to the 9.5 μl reactions (see Note 31). Incubate all samples (plus- and minus-reverse transcriptase reactions) for 30 min at 42°C. After cDNA synthesis, inactivate the Quantitect Reverse Transcriptase at 95°C for 3 min. Briefly centrifuge the Eppendorf tubes to remove drops from the inside of the lid. Continue with performing the quantitative real-time PCR or store the samples at −20°C until further usage.

21. Thaw wPRE and human β-Actin primers, plus- and minus-reverse transcriptase samples (see previous step) as well as water and 2× SYBR Green PCR Master Mix (both supplied with the Quantitect SYBR Green PCR Kit) on ice.
22. Prepare two independent master mix reactions for the detection of the RMT vector's wPRE element and the human β-actin cDNA containing each 7.5 μl 2× SYBR Green PCR Master Mix, 0.25 μl of each respective forward and reverse primer [15 pmol/μl] and 6.25 μl water per PCR reaction (see Note 32).
23. Start to preload the wells of a MicroAmp Fast Optical 96-Well Reaction Plate. To get triplicates, preload three wells for each sample with 14 μl/well of each the wPRE and the human β-actin master mix.
24. Add 1 μl sample to each of the designated wells and pipette up and down. In addition, include "no template controls" for wPRE and human β-actin (add 1 μl water instead of 1 μl sample) as well as 1:10 and 1:100 dilutions of one representative sample being positive for both target cDNAs (see Note 33).
25. Seal the plates with a MicroAmp Optical Adhesive Film and briefly (10 s) centrifuge at ~850 × *g* (centrifuge with rotatable tissue culture plate holders).
26. Subject the plate to the Real-Time PCR cycler and run the following program: Initial activation of the HotStarTaq DNA polymerase (part of the 2× Quantitect SYBR Green PCR Master Mix) for 15 min at 95°C. Proceed with 40 cycles: 95°C for 15 s, 60°C for 60 s and 72°C for 30 s. Include at the end of the program a melting curve analysis: 95°C for 15 s, 60°C for 60 s and 95°C for 15 s.
27. Determine relative retroviral genomic mRNA levels by applying the comparative $2^{-\Delta\Delta CT}$ method (see Notes 34 and 35).

3.5. Transduction of Target Cells with RMT Particles

As RMT particles just differ from typical gammaretroviral vector particles in the sequence of their PBS, the protocols used for the transduction of target cells are very similar. However, transduction protocols in general depend on the type of target cell (see Note 36). As an example, we will describe the RMT-mediated transfer of FLPo (codon-optimized FLP recombinase; (18)) into an adherent FLP reporter cell line derived from murine SC-1 fibroblasts (3, 15) (Fig. 2).

1. The day before transduction, seed 2.5×10^4 SC-1-derived FLP reporter cells per well of a 24-well plate.
2. Twenty-four hours later, prewarm complete DMEM culture medium to 37°C and thaw VSV-G-pseudotyped RMT.FLPo supernatants (generated as described in Subheadings 3.2 and 3.3) on ice.

3. Prepare transduction medium consisting of warm complete DMEM culture medium supplemented with protamine sulfate at a final concentration of 4 μg/mL.
4. Remove the used complete DMEM culture medium from seeded FLP reporter cells and add 500 μl transduction medium to each well.
5. Add different volumes of concentrated RMT.FLPo supernatant to the wells (e.g., 0.1 μl, 0.5 μl, 1 μl and 3 μl). Mix briefly by pipetting up and down with an adjustable micropipette.
6. Enhance transduction by centrifuging the plates at 863 × *g* and 30–37°C for 1 h (see Note 28).
7. Incubate the transduced cells for 6–14 h at 37°C with 5% CO_2.
8. Remove the virus-containing transduction medium and add 1 mL of fresh complete DMEM culture medium to the cells.
9. Determine the recombination efficiencies by performing FACS (fluorescence-activated cell sorting) analysis 36–48 h post transduction (see Note 37).

4. Notes

1. Codon-optimization adapts the tRNA codon usage of a given transgene sequence to highly favored codons for Homo sapiens. As a result in most cases the RNA is better transcribed, processed and translated in the cytosol. Simultaneously, we also ensure deletion of cryptic splice sites and polyadenylation motifs as well as the insertion of a proper Kozak consensus sequence for enhanced initiation of translation (19).
2. The insertion of the wPRE element in the 3′UTR of retroviral vectors greatly enhances vector titers as well as transgene expression in a variety of target cells. It increases vector RNA levels in cells and was shown to act on both spliced and unspliced RNAs at a posttranscriptional level (3, 9, 20, 21). Although the exact mechanism of the wPRE still needs to be elucidated, its function as a nuclear *cis*-acting RNA export and polyadenylation enhancer element could be demonstrated (10, 11, 22).
3. Heat-inactivation of the FCS is not necessarily required for cell cultivation but indispensable for retroviral particle production. Therefore, thaw and incubate the serum for 30 min at 56°C in a water bath before use. This inactivates potential antibodies as well as factors of the complement system, which recognize retroviral particles and impede their infectivity. However, avoid heat-inactivation of the FCS for more than 30 min, since this is counterproductive for particle production and/or target cell transduction.

4. We usually culture 293T, HeLa and SC-1-based FLP reporter cells in T-75 culture flasks. However, when expanding 293T cells for retroviral particle production (see Subheading 3.2) we usually use T-175 culture flasks. One confluent grown T-175 flask usually yields in a sufficient number of 293T cells for the seeding of eight to ten 10 cm-culture dishes.
5. The utilization of other viral envelopes/glycoproteins is also possible. We made good experiences with glycoproteins from the ecotropic Murine Leukemia Virus (Eco-Env; (23)) or a modified glycoprotein variant derived from feline endogenous retrovirus (RD114-TR; (24)). Be aware of that the type of the envelopes/glycoprotein determines the host tropism. For plasmid quantities and host tropism (see Table 1).
6. The pCMV-DsRed-Express (BD Biosciences Clontech) is included to control for transfection efficiencies and can be replaced by pEGFP-C1 (BD Biosciences Clontech, Heidelberg, Germany) or other nonviral expression plasmids, lacking the retroviral packaging signal Ψ.
7. For ultracentrifugal concentration, ideally, 32–34 mL of each freshly produced and filtrated supernatant should be provided. If less than 32 mL are available, always adjust with TFM to a final volume of at least 32 mL per polyallomer tube, otherwise the polyallomer tubes will collapse during the ultracentrifugation run.
8. The resuspension medium can be different from TFM. Usually, we resuspend the retroviral pellets in the culture medium of the target cell population.
9. Bacillol Plus is used here for inactivation of the VSV-G pseudotyped gammaretroviral particles remaining in the supernatant after ultracentrifugation. Other appropriate solvents or bleach (sodium hypochlorite) can be used for decontamination instead. Be aware of that the type of decontamination is dependent on the particles' pseudotype (envelope/glycoprotein). Also check the guidelines of your institution for appropriate inactivation of the respective particles.
10. Since 75% ethanol is used for washing extracted RNAs, only RNase- and DNase-free water should be used for dilution of 100% ethanol. Of note, we usually keep all solutions for RNA preparation separately.
11. If RMT particle quantification is performed on murine cells, use the following primer pair designed for the detection of murine β-actin: 5′-CCTCCCTGG AGAAGAGCTA-3′ (Forward) and 5′-TCCATACCCAAGAAGGAAGG-3′ (Reverse).
12. The RMT.FLPo vector particles are produced and concentrated as described in the methods chapter (Subheadings 3.2 and 3.3). We recommend the usage of concentrated (see Subheading 3.3)

RMT supernatants in general, since this results in high RMT efficacies at relatively low input volumes (Fig .2).

13. Surface-treated 24-well culture plates can be used instead. However, reduce the cell number from 5×10^4 to 2.5×10^4 per well when seeding the plates.
14. At the time of transfection, cells should be ~50–80% confluent. If cytotoxic transgenes (e.g., CRE recombinase) or envelopes/glycoproteins (e.g., VSV-G) are used, one should aim for a higher cell density (70–80% rather than 50%).
15. When performing double or even multiple transfection reactions we recommend using a 2 mL serological pipette for air bubbling.
16. Even though using surface-treated 10 cm-culture dishes, 293T cells tend to easily detach from the plates, especially when they are transfected. Thus, carefully add the medium to the dishes by using the electronic pipette filler at low speed.
17. 293T cells are very sensitive and should not be left without medium for long periods of time. Keep this in mind when performing multiple transfections. Thus, one plate should be handled after the other.
18. Chloroquine is a drug that prevents lysosomal acidification, thereby inhibiting the activation of lysosomal enzymes such as DNases. It is therefore included in the TFM during transfection to facilitate cytoplasmic persistence and nuclear entry of the transfected plasmids. However, excessive exposure of cells (>16 h) to chloroquine is cytotoxic and should be therefore avoided.
19. The 36 h- and the 60 h- supernatants not necessarily need to be combined and can be used separately, as it is also usual for most of the integration-competent retroviral particle preparations. The supernatants can be aliquoted and stored at −80°C until titration. However, since RMT is a transient method that transfers just two mRNAs per particle, concentration of the supernatants is beneficial. The concentration protocol described here (Subheading 3.3) requires ideally a final input volume of 32–34 mL per polyallomer tube. That is the reason why we usually perform double transfections and combine supernatants of two different time points to obtain a final volume of ~40 mL.
20. When concentration of the retroviral supernatants will be performed not later than 2 days after the last harvest, we usually keep them at 4°C (refrigerator) rather than freeze them at −80°C. By doing so, we expect to circumvent the loss of infectious particles that is described for freeze and thaw cycles of retroviral supernatants (25, 26).
21. If you have stored the in Subheading 3.2 produced RMT supernatants at −80°C, thaw them in the refrigerator overnight. This is convenient and allows gently thawing of the supernatants.

22. Before use, rinse rotor buckets with 80% ethanol and let them dry under sterile conditions. To save time, buckets can be also wiped out with a clean paper towel soaked in 80% ethanol.

23. To avoid particle destruction, ecotropic, VSV-G- and RD114-TR-pseudotyped particles are concentrated overnight at 13,238 × *g* (10,000 rpm using rotors SW28 or SW32Ti) and 4°C. However, VSV-G-pseudotyped particles can alternatively be concentrated using a short-term protocol at 82,740 × *g* (25,000 rpm using rotors SW28 or SW32Ti) and 4°C for 90 min.

24. Polyallomer tubes can be cleaned after usage. Decontaminate twice with Bacillol Plus solution for 15 min and rinse twice with 1× "Solution 555" (catalog number 339555, Beckman Coulter). Consider the waste solutions as S2 and dispose appropriately. Be aware of that "Solution 555" is a detergent. Thus, rinse decontaminated polyallomer tubes several times with distilled water until all traces of "Solution 555" have been removed. Dry and autoclave the tubes. Of note, if tubes are placed in empty pipette tip boxes, their deformation during autoclaving is avoided.

25. To obtain valid quantitative real-time PCR data, it is necessary to normalize the input of reverse transcribed sample RNA to a cellular housekeeping RNA (e.g., β-Actin). Retroviral particles contain in addition to their mRNA genome ~30 tRNA molecules and other small cellular RNAs (27–29). However, since the composition of cellular RNAs might be quite variable within produced retroviral particles, we recommend therefore performing titrations on transduced cells rather than on produced retroviral supernatants.

26. When selecting the cell line for particle titration, the viral tropism determined by the retroviral envelope/glycoprotein should be considered, e.g., ecotropic particles only infect murine and closely related rodent cells, and VSV-G all types of mammalian cells (see Table 1).

27. Protamine sulfate is a cationic polymer that cross-links equally charged retroviral particles and the target cells, thereby enhancing retroviral transduction efficiencies (30, 31).

28. Like protamine sulfate, centrifugal inoculation (spinoculation) was shown to effectively enhance retroviral infection of cultured cells (32). Until now, spinoculation is also a common method to improve the retrovirus-based transfer of genes into target cells (retroviral transduction) (33, 34). However, the underlying mechanisms remain poorly understood. Early studies suggest increased and more stable binding of the retroviral particle to its cellular surface receptor (35). Alternatively, Guo et al. suggest that spinoculation triggers dynamic actin and cofilin remodelling thereby facilitating the cellular entry of viral particles (36).

29. The volume of water used for dissolving the RNA depends on the pellet size. For cells and cell numbers given in the protocol here, we made good experience in dissolving the RNA in 30–60 μl (sometimes even 80 μl) water. When you are uncertain how much water to use, we recommend carefully increasing the amount until you achieve the correct volume. We suggest starting with a volume of 30 μl followed by incubation on a Thermo-shaker for 10 min at 50°C. If the RNA has not completely dissolved after incubation, add another 15–30 μl of water to the sample and incubate again. Repeat if necessary.

30. The TURBO DNase needs to be removed from the sample prior reverse transcription. Ambion recommends, inter alia, performing phenol/chloroform extraction. However, we made good experience with the RNAeasy Mini Kit from Qiagen for DNase removal.

31. In contrast to genomic DNA, the degradation of plasmid DNA by DNases is less efficient. Samples that lack the reverse transcriptase enzyme primarily therefore serve as controls for insufficient removal of plasmid DNA contaminations from the RNA sample. These controls are included during quantitative real-time PCR analysis and should ideally result in absent or very weak signals when compared to the corresponding reverse transcribed sample.

32. The master mix reactions should preferably be prepared in a room entirely free of plasmids to avoid interfering plasmid contaminations.

33. Serial dilutions (e.g., 1:10 and 1:100) of a representative sample are needed for calculating relative mRNA copies per sample via the comparative $2^{-\Delta\Delta CT}$ method (see also Note 34).

34. The comparative $2^{-\Delta\Delta CT}$ method is a mathematical way to quantify the relative changes in gene expression levels from real-time quantitative PCR experiments (37, 38). First, determine for each sample the average C_T values for the wPRE (avC_{T}-wPRE) and the human β-actin (avC_T-β-actin) cDNA. Second, subtract for each sample "avC_T-β-actin" from "avC_T-wPRE" to obtain the "ΔC_T" value. Third, calculate the average ΔC_T (avΔC_T) from "ΔC_T" values obtained from the undiluted and diluted (1:10 and 1:100) aliquots of a representative sample (see Subheading 3.4 step 24 and Note 33). Fourth, calculate for each sample the "$\Delta\Delta C_T$" value by subtracting the "avΔC_T" from the respective "ΔC_T" values. Finally, calculate for each sample the "$2^{-\Delta\Delta CT}$" values. The resulting data provide a relative measure for viral mRNA genome levels of entry-competent particles within RMT supernatants.

35. For the determination of absolute retroviral genomic mRNA levels of a given supernatant, a plasmid standard in serial dilutions

encoding the wPRE and the human β-actin cDNA template has to be included during real-time PCR analysis. However, mathematical methods other than the $2^{-\Delta\Delta CT}$ method need to be applied for analyzing absolute expression levels (39).

36. For the transduction of hematopoietic stem or progenitor cells via retroviral vector particles, we recommend retronectin-based transduction protocols (40). Retronectin is a recombinant human fibronectin fragment that enhances retrovirus-based gene transfer into human and murine cells expressing integrin receptors VLA-4 and/or VLA-5 on their surfaces (41–45). Retronectin is thought to colocalize target cells and retroviral particles by direct binding of cellular VLA-4 and/or VLA-5 and retroviral particles (46). Retronectin is composed of three functional domains that are responsible for the cross-linking function: The cell-binding domain and the CS-1 site bind to cellular VLA-5 and VLA-4 integrins, respectively, whereas, the third domain, the heparin-binding domain, is responsible for binding the retroviral vector particles. Retronectin can be purchased from Takara Bio Inc (catalog number T100A/B).
37. The ideal time point for analysis depends on the half-life of the transferred mRNA and the corresponding protein of interest, as well as on the read-out system. For example, EGFP expression in murine SC-1 cells transferred via RMT starts 5–7 h post transduction and reaches maximum levels at approximately 22 h (1, 2). Thus, the time point for FACS analyzing these cells should not exceed 22–24 h. In contrast, in case of RMT-mediated transfer of FLPo recombinase into a specially created FLP reporter cell line (Fig. 2), as described here, FACS analysis can be performed significantly later (>22–24 h). This is due to the fact that FLPo is only needed for a short duration to recombine the FLP reporter allele shown Fig. 2b and to activate permanent dTomato expression.

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Chapter 11

mRNA Delivery to Human Dendritic Cells by Recombinant Yeast and Activation of Antigen-Specific Memory T Cells

Frank Breinig, Tanja Breinig, and Manfred J. Schmitt

Abstract

The import of functional nucleic acids like messenger RNA into mammalian cells has proven to be a powerful tool in cell biology and several delivery systems have been described. However, as targeting of particular cell types is a major challenge and RNA vaccination represents a promising means for the induction of cellular immune responses, there is a need for novel delivery systems that permit the introduction of functional messenger RNA to the cytosol of immune cells. Here, we describe a delivery system based on the yeast *Saccharomyces cerevisiae* that allows the delivery of functional messenger RNA to mammalian antigen-presenting cells such as human dendritic cells. Further, we present a method to prove antigen processing and presentation by stimulation of human autologous T lymphocytes.

Key words: mRNA delivery, Antigen-presenting cell, T cell activation, *Saccharomyces cerevisiae*, Yeast

1. Introduction

Vaccination represents one of the most important means for prevention of infectious diseases. However, there is a lack of vaccines against several pathogens, among them major threats like HIV or HCV; therapeutic vaccines for use in cancer treatment are also still missing. Thus, the development of effective, economic and safe vaccines is a major challenge in todays medicine. In particular, there is a need for vaccines that efficiently induce CD8-positive cytotoxic T lymphocytes (CTL) which are essential to resist several bacterial and many viral infections as well as to eliminate tumor cells, making the induction of CTL an important goal in current vaccination strategies. As the efficient induction of CTL requires presentation of antigen-derived peptides via the major histocom-

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patibility complex class I (MHC I)-pathway, the antigen of interest should ideally be produced within the cytosol of an antigen-presenting cell (APC). In view of that, DNA/RNA vaccination has become the most promising strategy for inducing cell-mediated immunity (1, 2) and several approaches have been described reaching from classical DNA vaccination up to the delivery of antigen-encoding DNA or RNA by attenuated viruses or bacteria. In the literature, the delivery of messenger RNA (mRNA) instead of DNA is favored as (1) it avoids the safety problem of integration of plasmid DNA into the host cell genome that arises as a result of DNA vaccination, (2) it circumvents the need of plasmid DNA entry into the nucleus for high-level gene expression, and (3) it makes the use of mammalian promoters dispensable, a fact that can at least diminish the possibility of dangerous side effects (3).

Most of the bacterial strains that have been used for antigen-delivery belong to the large group of invasive enteric bacterial pathogens such as *Escherichia coli*, *Shigella*, *Salmonella*, or *Listeria*. Hitherto, several of these attenuated bacteria have been exploited to deliver heterologous antigens to mammalian cells as functional mRNA to be expressed in the infected host cell. However, although a number of studies have indicated the principle feasibility of this approach, convincing results of clinical trials are still missing (4). Furthermore, bacteria often contain highly toxic cellular components such as lipopolysaccharides (LPS, gram-negative bacteria) or super antigens (many gram-positive bacteria) which detract them from being used in humans for safety concerns or at least diminish the acceptance of a putative vaccine. Further, the induction of a vector-neutralizing immune response is observed in many cases when using the same vector system for repeated vaccinations.

Per se, several attributes qualify yeast as novel, promising mRNA vaccine candidate: yeast is known to elicit adjuvant effects by stimulating innate immune responses by structural cell wall components and to enhance cross-presentation of antigens (5–7). Yeast has been repeatedly shown to generate adaptive (including cell-mediated) immune responses in mice (8–11) and also in humans (12). In contrast to the majority of attenuated bacteria and mammalian viruses (which are already under investigation as mRNA carriers) several yeast genera possess the GRAS ("generally regarded as safe") status which would facilitate the approval of a yeast-based vaccine considerably. It is devoid of endotoxins and super antigens, and most strains are, due to the tremendous experience in using yeast in genetics and biotechnology for many years, accessible to genetic modifications allowing the development of multivalent vaccines and/or the modulation of immune responses. Yeast, although being a genuine eukaryote, harbors the advantages of a single-cell microorganism which is easy and safe to handle (i.e., small size, easy cultivation, fast cell growth). Moreover, recombinant yeast can be given orally as yeast cells are able to protect

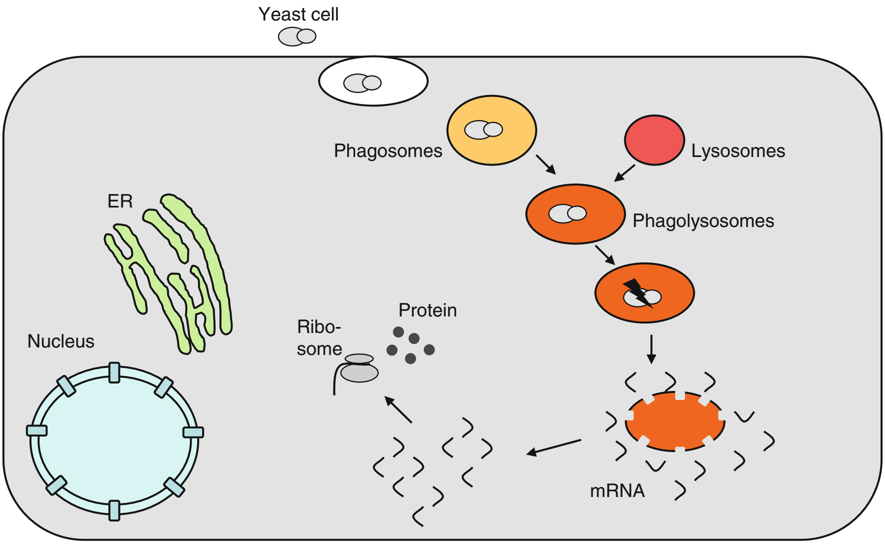

Fig. 1. Principle of yeast-mediated mRNA delivery. After phagocytosis of yeast cells by the particular APC, the resulting phagosomes merge with lysosomes. Within these phagolysosomes, yeast cells are degraded and mRNA molecules are released into the cytosol of the APC by a so far unknown mechanism. The delivered mRNA can be translated directly by ribosomes within the mammalian cell yielding the protein-of-interest.

the antigen-encoding nucleic acid from degradation by acidic pH and/or nucleases during passage through stomach. Furthermore, yeast cells are efficiently taken up by M cells, located in gut associated lymphoid tissue (13), and may subsequently deliver their nucleic acid load to professional APCs in Peyer's patches, an important prerequisite for the induction of a mucosal immune response.

Recently, we reported the delivery of functional "transgenic" mRNA to mammalian APCs using the yeast *Saccharomyces cerevisiae* as a delivery vehicle ((14); see Fig 1 for an overview). The newly developed system relies on the fact that yeast cells are taken up effectively by professional APCs like macrophages and dendritic cells (DCs). The particular yeast cells either accumulated mRNA before uptake by the APCs (constitutive mRNA expression) or start mRNA transcription after uptake (inducible mRNA expression). The latter is achieved by using promoters from genes involved in the glyoxylate cycle which have been shown to be activated in phagosomes after engulfment of yeast cells by macrophages (15). Translation of transgenic mRNA in yeast is prevented by addition of a viral internal ribosomal entry site (IRES) that, due to the lack of specific translation-initiating factors, can not be recognized in yeast (16). However, when mRNA being released from yeast, IRES enables mRNA translation in cytosol of mammalian APCs. This system was established by reprogramming murine macrophages and human DCs with eGFP mRNA and then used for reprogramming human DCs to enable activation of human memory T cells directed against the tegument protein pp65 from cytomegalovirus (CMV) (14).

The use of recombinant yeast system for mRNA delivery is a novel approach in mRNA vaccination strategies and a promising alternative to another methods such as viral and bacterial delivery systems. In this context, an exclusive advantage of yeast-mediated delivery consists in the specific targeting of the delivered nucleic acid to phagocytic APCs. In contrast, routine methods of mRNA delivery are usually unspecific. The majority of carriers currently under investigation (bacteria and viruses) is unable to target APC in particular and rather infects a variety of cell types like epithelial, muscle cells and other non-APCs (2, 17, 18).

mRNA-transfer from phagolysosomes to the cytosol of APC takes place without the need of additional and potentially toxic components destabilizing endosomal/phagosomal membranes. In contrast to known bacterial or viral systems, yeast cells do neither interfere with cell growth nor cell function of mammalian APC (19–21).

So far, the mechanism of mRNA transfer from phagolysosomes to the cytosol is completely unknown. A simple rupture of the phagolysosomes seems unlikely as such an event would release lysosomal activity which, as a consequence, would presumably affect the viability of the APCs. However, there are several reports in which proteins delivered by recombinant yeast are capable of entering in particular the MHC I pathway via cross-presentation (8, 10, 12, 19, 22), a mechanism whose molecular basis is currently controversely discussed. One explanation for this phenomenon could be the observation that phagosomes containing exogenous proteins merge with the endoplasmic reticulum (ER) in professional APCs such as macrophages and DCs. Subsequently, the proteins are capable of exiting the ER via pores that are normally involved in ER protein quality control (ERAD, *e*ndoplasmic *r*eticulum *a*ssociated *d*egradation), enter the cytosol and reenter the ER as peptides through binding to MHC I molecules (23). This raises the possibility that mRNA molecules from internalized yeast cells might be able to exit phagosomes and/or the ER either unspecifically by hijacking cellular ERAD components or enter the cytosol through a specific but so far unknown mechanism to initiate subsequent translation. Finally, another attractive feature of the yeast-based system lies in the fact that clearly no neutralization (i.e., the induction of an immune response against the carrier itself which hampers repeated vaccination with the same delivery vehicle) of the vector system occurs even after repeated immunizations (24), a well-known phenomenon which so far has hindered the use of several viral as well as bacterial vector systems. In conclusion, the yeast-based delivery system offers several advantages in comparison to established bacterial and viral carrier systems. It represents a promising and innovative approach whose potential for vaccination and other clinical applications has to be elucidated in the near future.

First part of the protocol (Subheadings 3.1–3.3) describes the construction of yeast vectors containing GFP reporter gene and transfer of GFP mRNA to murine IC21 cells. The macrophage-like cell line IC21 is used to work out the method as it represents a simple mammalian APC that is easy to culture and available in high numbers. The second part (Subheading 3.4/Subheading 3.5) provides instructions for isolation, differentiation and transfection of human dendritic cells, the most important human APC. As these are primary cells they are only available in limited numbers and require a optimized transfection protocol achieved in the first part. Finally, combining the protocols from parts one and two, we provide a method for modulation of human dendritic cells by yeast-mediated delivery of antigen-encoding mRNA and activation of human autologous antigen-specific T cells (Subheading 3.6).

2. Materials

2.1. Equipment

1. Thermocycler.
2. Agarose gel chamber with power supply.
3. General purpose centrifuge.
4. Flow cytometer.

2.2. Cells and Plasmids

1. *Escherichia coli* TOP10 [F^- *mcr*A Δ(*mrr-hsd*RMS-*mrc*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rsp*L (Str^R) *end*A1 *nup*G] [(Cat number C4040-03) (Invitrogen); see Note 1].
2. *Saccharomyces cerevisiae* S86c [MATα *ura3-2 his3 pra1 prb2 prc1 cps1* L-0M-0] (see Note 2).
3. Murine macrophage-like cell line IC21 (see Note 3).
4. Human monocyte-derived DC (see Note 3; for preparation see Subheading 3.4).
5. Yeast expression vector pPGK-M28-I (see Note 4).
6. Mammalian expression vector pIRES (Cat number 631605) (Clontech) (see Note 5).
7. Mammalian expression vector pEGFP-C1 (Cat number 632406) (Clontech).
8. pp65-encoding vector jw4303 (see Note 4).

2.3. Growth Media

Grow *E. coli* TOP10 cells containing pPGK-M28-I or a delivery plasmid at 37°C in standard LB medium supplemented with 50 μg/ml ampicillin for selection of the particular plasmid. Grow yeast cells containing pPGK-M28-I or a delivery plasmid at 30°C in synthetic medium (YNB) without uracil for selection of the particular plasmid or non-transfected cells in standard YEPD medium. The murine macrophage-like cell line IC21 is cultured in standard RPMI 1640 medium with L-glutamine supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% (v/v) CO_2 atmosphere. For cultivation of human monocyte-derived DC see below (see Subheading 3.4).

2.4. Construction of Delivery Plasmids

1. Standard PCR reagents.
2. Agarose.
3. 10× TBE Stock: 108 g Tris base, 55 g boric acid, 9.3 g EDTA (pH 8.3), adjust volume to 1 L with distilled water (Stock, store at room temperature).
4. 1× TBE working solution: dilute the 10× stock 1:10 with distilled water.
5. Restriction enzyme *Xho*I.
6. Restriction enzyme *Bgl*II.
7. Restriction enzyme *Hin*dIII.
8. Restriction enzyme *Sac*I.
9. GFP primer Fw (see Note 6):

   5′[GAAAAACACGATGATAAGCTTGCCACAATGGTGAGCAAGGGCGAGGAGC]
10. Primer GFP Rv:

    5′[*AGATCT*TTACTTGTACAGCTCGTCCATGCCGAG]
11. Primer IRES Fw:

    5′[*CTCGAG*GCCCCTCTCCCTCCCCCC]
12. Primer IRES Rv:

    5′[GCTCCTCGCCCTTGCTCACCATTGTGGCAAGCTTATCATCGTGTTTTTC]
13. Primer SV40polyA Fw:

    5′[*AGATCT*TCTAGAATAATCAGCCATACCACATTTGTAGAGGTTTTACTT]
14. Primer SV40polyA Rv:

    5′[*GAGCTC*CAGACATGATAAGATACATTGATGAGTTTGGACA]

2.5. Transformation of Yeast Cells

1. YEPD medium.
2. YNB medium without uracil.
3. YNB plates without uracil.
4. 10× LiAc Stock: 1 M lithium acetate in distilled water (Stock, store at room temperature).

5. 10× TE Stock: 100 mM Tris base, 10 mM EDTA, adjust pH to 7.5 with HCl (Stock, store at room temperature).
6. LiAc/TE: dilute 10× TE and 10× LiAc 1:10 in distilled water (just before use).
7. PEG 4000 (Stock): 50% PEG 4000 in distilled water (Stock, store at room temperature).
8. PEG solution: 80% PEG 4000 stock, 10% 10× TE, 10% 10× LiAc (just before use).
9. Salmon sperm DNA (10 mg/ml): denature in boiling water for 15 min, keep at –20°C until use.
10. Sterile 1.5 ml tubes.

2.6. Loading of APC with Yeast Cells and Generation/Staining of Human Monocyte-Derived DC

1. Standard buffy coat preparations of healthy blood donors (available for example from your local hospital) or fresh blood (see Note 7).
2. Lymphocyte separation medium LSM 1077 (Cat number J11-004) (PAA).
3. LPS-free heat-inactivated Fetal Calf Serum (LPS-free FCS).
4. Heat-inactivated standard Fetal Calf Serum (sFCS).
5. PBS (without calcium and magnesium (see Note 8)).
6. FACS-FIX: NaCl 8.5 g/L, paraformaldehyde 10 g/L, adjust pH to 7.4 (store at 4°C).
7. GM-CSF.
8. IL-4.
9. Anti-CD1a-FITC (Clone NA1/34) (Cat number F7141) (DAKO).
10. Anti-CD14-PE (Clone TUEK4) (Cat number R0864) (DAKO).
11. Anti-HLA-DP, DQ, DR-FITC (Clone CR3/43) (Cat number F0817) (DAKO).
12. Anti-HLA-A, B, C-PE (Clone W6/32) (Cat number R7000) (DAKO).
13. Anti-CD80-PE (Clone MAB104) (Cat number IM1976U) (Beckman Coulter).
14. Anti-CD83-PE (Clone HB15a) (Cat number IM2218U) (Beckman Coulter).
15. Anti-CD86-FITC (Clone 2331) (Cat number 555657) (Becton Dickinson).
16. 24 well-plates.
17. Cell culture flasks (75 cm^2).
18. Cell culture flasks (175 cm^2).

19. Disposable pipettes.
20. Polypropylene 50 ml centrifuge tubes.
21. FACS tubes.

2.7. Stimulation and Staining of Antigen-Specific T Cells

1. Primer IRES-pp65 Fw:

   5′[GAAAAACACGATGATAAGCTTGCCACAATGATATCCGTACTGGGTCCCATTTCG]
2. Primer IRES-pp65 Rv:

   5′[CGAAATGGGACCCAGTACGGATATCATTGTGGCAAGCTTATCATCGTGTTTTTC]
3. Primer pp65 Rv:

   5′[*AGATCT*TCAACCTCGGTGCTTTTTGGGC]
4. Polypropylene 15 ml centrifuge tubes.
5. Cryogenic tubes.
6. Recombinant pp65 (Cat number 130-091-823) (Miltenyi Biotec).
7. Anti-CD28 (non-labelled) (Cat number 348040) (Becton Dickinson).
8. Anti-CD49d (Clone 9F10, non-labelled) (Cat number 555501) (Becton Dickinson).
9. Anti-CD69-PE (Clone TP1.55.3) (Cat number IM1943U) (Beckman Coulter).
10. Anti-IFNγ-FITC (Clone 4S.B3) (Cat number 554551) (Becton Dickinson).
11. Anti-CD4-PC5 (Clone 13B8.2) (Cat number A07752) (Beckman Coulter).
12. Anti-CD8-PC5 (Clone B9.11) (Cat number A00758) (Beckman Coulter).
13. Brefeldin A Stock 5 mg/ml in DMSO (Stock, keep at 4°C).
14. Brefeldin A working solution: dilute the stock 1:10 with PBS (just before use).
15. 20 mM EDTA (keep at room temperature).
16. 0.02% EDTA in PBS (keep at room temperature).
17. FACS buffer: 500 ml PBS, 25 ml sFCS, 2.5 g BSA, 1.4 ml 25% Na-Azide.
18. Saponin (Cat number 47036-50G-F) (Sigma Aldrich).
19. 4% paraformaldehyde (see Note 9).

3. Methods

3.1. Construction of Delivery Plasmids

The gene-of-interest is inserted as IRES fusion in the yeast expression vector pPGK-M28-I (25) by standard cloning methods (see Fig. 2). The following procedure describes the construction of a plasmid for delivery of mRNA encoding GFP as reporter protein.

1. Use "Splicing by Overlapping Extension" (SOE) PCR (see Note 10) to add an "internal ribosomal entry site" (IRES) upstream of the GFP gene (see Note 5). Perform two standard PCR reactions to amplificate GFP (Template pEGFP-C1; primers GFP Fw and GFP Rv) and to amplificate IRES (Template pIRES; primers IRES Fw and IRES Rv).
2. Isolate the resulting products from agarose gel (1% agarose in 1× TBE, running buffer: 1× TBE) and use these products as template in a third PCR using primers IRES Fw and GFP Rv, yielding the IRES-GFP fusion as well as 5′-*Xho*I and 3′-*Bgl*II restriction sites.
3. Sequence the resulting fragment and subclone into pPGK-M28-I after restriction with *Xho*I/*Bgl*II yielding pPGK-IRES-GFP (see Notes 11 and 12).
4. Perform a PCR reaction to amplificate the polyadenylation signal sequence (225 nucleotides) from simian virus 40 (SV40; template pEGFP-C1; primers SV40polyA Fw and SV40polyA Rv) yielding the SV40 polyadenylation signal sequence as well as 5′-*Bgl*II and 3′-*Sac*I restriction sites.
5. Sequence the resulting fragment to confirm its correct sequence and subclone into pPGK-IRES-GFP after restriction with *Bgl*II/*Sac*I (see Note 13).
6. Transfect *S. cerevisiae* strain S86c with the resulting delivery plasmid (see Note 14).

3.2. Transformation of Yeast Cells

1. Inoculate a single colony of *S. cerevisiae* S86c in 5 ml YEPD medium.
2. Grow on shaker to $OD_{600} = 1$ (OD_{600} between 0.8 and 1.6 will do) overnight at 30°C.
3. Transfer 2 ml of the culture in a sterile 1.5 ml tube and centrifuge for 5 min at 3,000 × *g* at room temperature.
4. Wash cells once with 500 μl LiAc/TE.
5. Resuspend pellet in 200 μl LiAc/TE, add consecutively 15 μl Salmon sperm carrier DNA (10 mg/ml), 600 μl PEG solution, 50 μl 10× LiAc, and 2 μl plasmid DNA (~1–2 μg DNA) and mix.
6. Incubate on shaker for 1 h at 30°C.

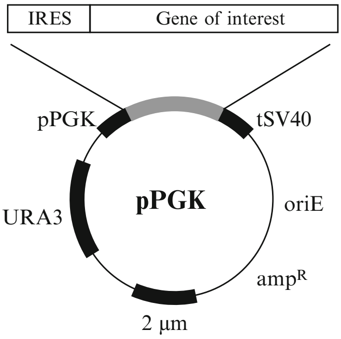

Fig. 2. Schematic drawing highlighting the features of a plasmid for yeast-mediated mRNA delivery. The plasmid represents a typical shuttle vector containing origins of replication as well as selectable markers for both *E. coli* (oriE, amp^R) and *S. cerevisiae* (2 µm, *URA3*). Further, it possesses a yeast-based promoter (e.g., *PGK*), an expression cassette consisting of an IRES/gene-of-interest-fusion and the SV40 polyadenylation signal.

7. Heat shock for 15 min at 42°C.
8. Centrifuge cells for 5 min at 3,000 × *g* and wash twice with 500 µl 1× TE.
9. Resuspend cells in 200 µl 1× TE, plate on YNB plates without uracil and incubate for at least 3 days at 30°C.

3.3. Loading of Murine Macrophages with Yeast

See Fig. 3a for a typical result after delivery of eGFP-encoding mRNA to murine macrophages. All centrifugations are carried out at 4°C unless otherwise stated.

1. Seed IC21 macrophages 8 h prior loading into 24-well plates at a density of 1×10^5 cells per well in standard RPMI medium supplemented with 10% heat-inactivated sFCS.
2. Add yeast cells (cultured until $OD_{600} = 1$ in YNB medium without uracil) at MOI 5 (see Note 15) in 2 ml medium and coculture for 16 h (see Note 16). Centrifuge for 5 min at 300 × *g* to bring yeast cells and APC together and to enhance uptake of yeast cells.
3. Confirm successful uptake of yeast cells via light microscopy (see Note 14).
4. Remove the medium and wash the cells once with PBS.
5. Determine successful GFP expression via flow cytometry (see Fig. 3a, and Notes 17 and 18).

3.4. Preparation of Human DC

Human monocytes are isolated from PBMC ("peripheral blood mononuclear cells") and then differentiated to DC by cultivation in the presence of the cytokines GM-CSF and IL-4. All centrifugations are carried out at 4°C unless otherwise stated.

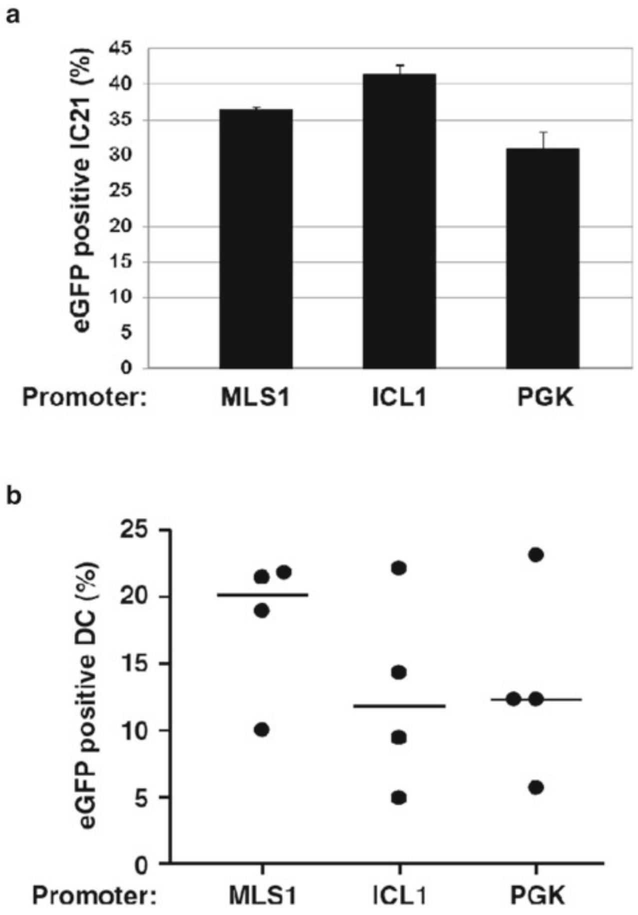

Fig. 3. Yeast-based delivery of mRNA to mammalian APC. Yeast cells delivering eGFP-encoding mRNA under control of different promoters were added to IC21 macrophages (**a**) or human DCs (**b**) at MOI 7 or MOI 5, respectively. The number of eGFP-positive cells from 4 independent blood donors was measured 16 h p.i. by flow cytometry. The bar indicates the median value. According to (14), modified.

3.4.1. Isolation of PBMC from Buffy Coats

1. Prepare 50 ml tubes: for each buffy coat set up four to six tubes containing 15 ml PBS and four to six tubes with 15 ml Ficoll (see Notes 7 and 8).
2. Fill each PBS-containing tube with 20 ml of blood; mix by inverting the tube.
3. Put the PBS–blood mixture *carefully* on the Ficoll (see Note 19).
4. Centrifuge for 25 min at 560 × *g* with *brake off*; complete running time approx. 1 h (see Note 20).
5. Remove and discard 10 ml of the supernatant above the ring layer of leukocytes (see Fig 4).

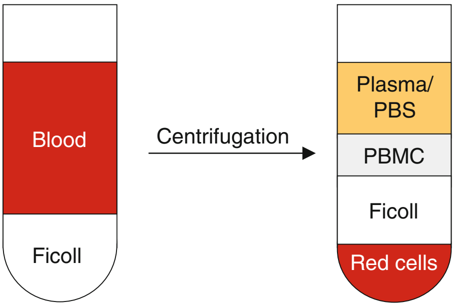

Fig. 4. Separation of whole blood within a Ficoll gradient.

6. Collect the ring of leukocytes using a 10 ml pipette tip in rotary motion and put the cells in a new 50 ml tube (see Fig 4 and Note 21).
7. Fill each tube with PBS (room temperature) up to 50 ml.
8. Centrifuge for 6 min at 500 × *g* (washing step) and remove the supernatant carefully.
9. Resuspend each pellet in 5 ml H_2O and refill the tube *directly* with PBS (removal of erythrocytes; see Note 22).
10. Centrifuge for 6 min at 500 × *g*.
11. Repeat until the pellet loses red color (usually 1×).
12. Pool the pellets of each buffy coat and resuspend the cells in 50 ml PBS.
13. Centrifuge for 10 min at 300 × *g* and remove the supernatant.
14. Resuspend the PBMC in 50 ml RPMI 1640 medium containing 10% sFCS and determine the cell number (see Note 23).

3.4.2. Isolation of Monocytes by Adherence (see Note 24)

1. Dilute PBMC to 7.5×10^6 cells per ml; use 20 ml of this suspension per 175 cm^2 cell culture flask.
2. Incubate for 2 h at 37°C and 5% CO_2.
3. Remove non-adherent cells and wash adherent monocytes twice with PBS.

3.4.3. Differentiation of Monocytes to Immature DC

1. Add 20 ml RPMI 1640 medium containing 10% heat-inactivated LPS-free FCS (see Note 25), 800 U/ml GM-CSF and 1,000 U/ml IL-4 per 175 cm^2 cell culture flask.
2. Incubate for 5 days at 37°C and 5% CO_2 (see Note 26).
3. At day 3, replace 5 ml medium by spinning down the cells and adding 5 ml fresh medium prepared according to step 1.

3.4.4. Characterization of Immature DC

Confirm successful preparation of immature DC by checking the cell surface expression of marker proteins via flow cytometry.

1. Put 5×10^5 cells per sample in a FACS tube.
2. Wash DC twice with PBS supplemented with 10% sFCS.
3. Add titrated amounts of labelled monoclonal antibodies (see Note 27) specific for the particular cell surface marker (see Subheading 2.6) and incubate for 45 min on ice in the dark (different direct labelled antibodies can be added together; for several samples use an antibody-mix). Prepare also one sample as isotype control (e.g., IgG1-FITC/IgG2a-PE, depending on the isotype of the antibody used).
4. Wash two times with PBS supplemented with 10% sFCS and discard the supernatant.
5. Resuspend the cells in 200 μl FACS-FIX and incubate for 30 min at 4°C in the dark.
6. Analyze at least 10,000 cells by flow cytometry (see Note 28).

3.5. Loading of Human DC with Yeast

See Fig. 3b for a typical result after delivery of eGFP-encoding mRNA to human DC. All centrifugations are carried out at 4°C unless otherwise stated.

1. Seed immature DC 8 h prior loading into 24-well plates at a density of 1×10^6 cells per well in RPMI medium supplemented with 10% sFCS.
2. Add yeast cells (cultured until $OD_{600} = 1$ in YNB medium without uracil) at MOI 5 (see Note 15) in 2 ml medium and coculture for 16 h (see Note 16). Centrifuge for 5 min at $300 \times g$ to bring yeast cells and APC together and to enhance uptake of yeast cells.
3. Confirm successful uptake of yeast cells via light microscopy (see Note 14).
4. Remove the medium and wash the cells once with PBS.
5. Determine successful GFP expression via flow cytometry (see Fig. 4b and Notes 17, 18, 29).

3.6. Stimulation of pp65-Specific Memory T Cells by Autologous DC

We delivered mRNA of the major CMV tegument protein pp65 allowing the stimulation of human pp65-specific memory T cells within the blood of seropositive donors. See Fig. 5a for an overview of the MHC-I-restricted antigen-specific T cell stimulation protocol and Fig. 5b for a typical result. Specifically activated CD4 or CD8 T cells are identified as CD69/IFNγ double-positive cells by flow cytometry. All centrifugations are carried out at 4°C unless otherwise stated.

3.6.1. Construction of pp65-Delivery Plasmid

1. Construct a pp65-delivering plasmid by SOE-PCR as described in Subheading 3.1 using plasmid jw4303 as template. Perform two standard PCR reactions to amplificate pp65 (primers IRES-pp65 Fw and pp65 Rv) and IRES (primers IRES Fw and IRES-pp65 Rv).

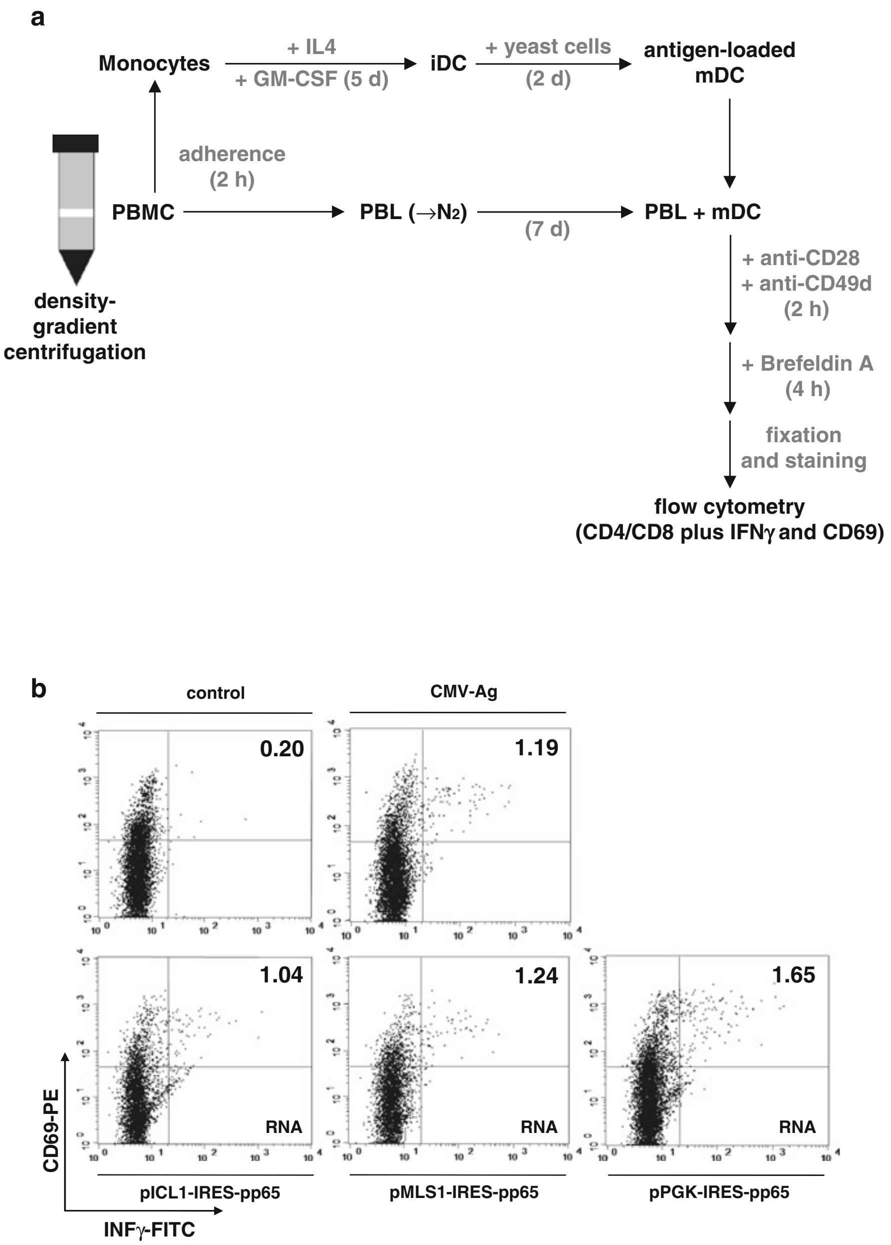

Fig. 5. Antigen processing and presentation after yeast-mediated mRNA delivery. *Experimental scheme for the antigen-specific stimulation of memory T cells by autologous DCs* (**a**). Peripheral blood mononuclear cells (PBMC) were separated into monocytes and lymphocytes (PBL) by adherence. PBL were kept frozen in liquid nitrogen (N_2) until use, whereas monocytes were differentiated into iDCs as described above. The iDCs were incubated for 48 h with recombinant yeast cells at MOI 5. The cells were then incubated together with the autologous PBL in an 1:1 ratio in the presence of antibodies against CD49d and CD28. After 2 h, Brefeldin A (BFA) was added to the cells to inhibit secretion of produced cytokines. After fixation, cells were stained for intracellular IFN-γ and the surface markers CD69 and CD4 or CD8 and subsequently analyzed by flow cytometry for simultaneous expression of stained markers [iDC: immature DCs; mDC: mature DCs]. *Delivery of pp65-encoding mRNA by recombinant yeast leads to an efficient activation of CD8-positive T cells by human DCs* (**b**). Yeast cells containing the indicated plasmid for mRNA delivery were added to human monocyte-derived DCs and activation of autologous, pp65-specific CD8 T lymphocytes was measured by staining of the early activation marker CD69 as well as the effector molecule IFN-γ. A CMV lysate (CMV-Ag) containing pp65 protein was used as positive control, a vector containing an irrelevant antigen (ovalbumin) served as negative control. Dot blots of a representative donor showing the frequency (upper right) of antigen-specific activated, CD69/IFN-γ double-positive human CD8 T cells are shown. According to (14), modified.

2. Isolate the resulting products from a standard agarose gel (1% agarose in 1× TBE, running buffer: 1× TBE) and use these products as template in a third PCR using primers IRES Fw and pp65 Rv yielding the IRES-pp65 fusion as well as 5′-*Xho*I and 3′-*Bgl*II restriction sites.
3. Sequence the resulting fragment and subclone into pPGK-M28-I after restriction with *Xho*I/*Bgl*II yielding pPGK-IRES-pp65.
4. Proceed as described in Subheading 3.1 for the construction of delivery plasmids.

3.6.2. Stimulation of T Cells

1. Isolate PBMC and separate monocytes according to Subheading 3.4.
2. Collect the supernatant with non-adherent cells after adherence (PBL, see Note 30) and freeze PBL in liquid nitrogen: centrifuge PBL for 5 min at 300 g, resuspend the cells in freezing medium (growth medium containing 10% DMSO) and transfer in cryogenic tubes. Keep tubes in liquid nitrogen until use.
3. Differentiate the monocytes to immature DC according to Subheading 3.4.3/Subheading 3.4.4.
4. Load the immature DC with recombinant yeast cells according to Subheading 3.5 but incubate for 48 h in order to mature DC and allow presentation of antigen-derived peptides. Use yeast cells delivering the empty vector as a negative control and titrated amounts of recombinant pp65 as positive control.
5. Transfer mature DC into polypropylene15 ml tubes (see Notes 31 and 32).
6. Add thawed autologous PBL from Subheading 3.5 step 2 in an 1:1 ratio to matured DC and incubate for 30 min at 37°C and 5% CO_2 in the presence of antibodies against CD49d and CD28 (1 μg/ml each) with loose cap (see Note 33).
7. Centrifuge tubes for 5 min at 300 × *g*.
8. Incubate tubes at 37°C and 5% CO_2 for further 90 min.
9. Add 10 μg/ml Brefeldin A (see Note 34), vortex, and incubate at 37°C and 5% CO_2 for further 4 h.

3.6.3. Fixation and Staining of the Cells (see Note 35)

1. Warm up 4% paraformaldehyde to 37°C.
2. Add 100 μl/ml 20 mM EDTA, vortex, and incubate for 15 min at room temperature.
3. Add 2 ml 0.02% EDTA in PBS and vortex.
4. Centrifuge for 6 min at 300 × *g* and discard supernatant.
5. Incubate cells for 5 min in 300 μl 4% paraformaldehyde at 37°C.

6. Add 2 ml FACS buffer (see Note 36) and centrifuge for 6 min at 300 × *g*.
7. Add titrated amounts of labelled monoclonal antibodies specific for surface marker CD69 and CD4 or CD8 (see Subheading 2.6) and stain as described in Subheading 3.4.4 (see Note 37).
8. Put 5×10^5 cells per sample in a FACS tube (see Note 38).
9. Add 1 ml PBS supplemented with 4% paraformaldehyde per sample and incubate for 30 min at room temperature (see Note 35).
10. Wash cells twice with FACS buffer.
11. Add 2 ml 0.1% saponin in FACS buffer for *exactly 10 min* (see Note 39) at room temperature and centrifugate for 6 min at 300 × *g*.
12. Add titrated amounts of labelled monoclonal IFNγ antibody (see Note 27) and incubate for 45 min on ice in the dark.
13. Wash two times with 0.1% saponin in FACS buffer.
14. Resuspend in 200 μl FACS-FIX and incubate for 30 min at 4°C in the dark.
15. Analyze at least 10,000 cells by flow cytometry (see Note 28).

4. Notes

1. For cloning and construction of delivery plasmids; you can also use another standard strain of your choice.
2. For use as vehicle to deliver the particular mRNA; If *S. cerevisiae* S86c is not available, you can also use another standard strain of your choice. If you do so, be aware to check the uptake of this strain by the particular APC in advance.
3. For use as host cell and detection of successful mRNA delivery; we recommend to use the murine IC21 cells for establishing your system and initial experiments before applying the method to human primary cells such as dendritic cells.
4. If yeast expression vector pPGK-M28-I and pp65-containing vector jw4303 (19) are not available, you can also use another yeast expression vector or any pp65-encoding vector of your choice, respectively.
5. The upstream IRES sequence prevents the mRNA from being expressed in yeast and enhances translation in mammalian cells. Encephalomyocarditis virus-IRES is incorporated in several commercially available mammalian expression vectors.

6. Restriction sites are shown in *italics* within the primer sequence. Replace the underlined sequences of the primers by the sequences of your gene-of-interest in order to construct delivery vectors for your particular gene.
7. Note that the volume of a buffy coat may vary. If there are no buffy coats available, use fresh blood; 1 ml whole blood contains approximately 1×10^6 PBMC.
8. Use PBS without calcium and magnesium as these ions stimulate the T cells unspecifically.
9. For 100 ml of a 4% Stock add 4 g paraformaldehyde powder to 100 ml PBS and heat to 70°C (do not exceed this temperature) until solution becomes clear. Cool down at RT, filter and store at 4°C protected from light.
10. Using SOE PCR you are able to combine several parts of different genes without introduction of restriction sites and cloning. It is based on two PCR reactions that yield products with overlapping ends at the 3′ or 5′ ends, respectively. Based on their complementary ends, these products serve in a third PCR as “self-templates” yielding a full-length product in which the two original products are joined and amplificated using the “end primers” (5′-primer from reaction 1 and 3′-primer from reaction two) of the desired full-length product. Adapt the underlined parts of the primers to the sequence of your particular gene-of-interest.
11. This double-restriction replaces the cDNA from the yeast K28 killer toxin (~1,000 bp) originally contained in pPGK-M28-I with the IRES-GFP fusion.
12. This kind of cloning inserts the IRES-GFP fusion behind the *PGK* promoter originally contained in pPGK-M28-I leading to constitutive mRNA expression within the yeast cell. However, the efficacy of different promoters for mRNA delivery can vary significantly depending on your gene of interest and the particular APC used (see Fig. 3). We recommend to test also inducible promoters (e.g., *MLS1* or *ICL1*) and compare their efficacy in your particular approach. The *PGK* promoter can be replaced by another promoter sequence by *Hin*dIII/*Xho*I double digest; yeast promoter sequences can be obtained from the *S. cerevisiae* promoter database (http://rulai.cshl.edu/SCPD). Be aware to replace the promoter region in the original pPGK-M28-I plasmid *before* insertion of your IRES-gene-of-interest fusion as the IRES sequence itself contains a *Hin*dIII restriction site.
13. In principle, the yeast-derived *PGK* terminator included in pPGK-M28-I is functional in mammalian cells. However, replacing by the SV40 poly(A) signal improved yeast-mediated delivery of nucleic acids (see ref. 14).

14. Based on their interaction with receptors on the surface of APC, different yeast strains can be taken up more or less efficiently (influencing directly the expression of your gene-of-interest) and even the uptake of the same strain varies sometimes between different experiments. Therefore, it is recommended to include always an "uptake control" in an extra well consisting of for example fluorescent yeast cells [expressing GFP or stained with 1 μg/ml fluorescein diacetate (Cat number F7378-5G) (Sigma Aldrich)] which gives a clue for uptake efficacy in the particular experiment. Do not use results from experiments in which less than 50% of the APC have taken up green fluorescent control yeast cells.
15. MOI indicates the ratio of yeast cells to APC, e. g. MOI 5 means that 5× more yeast cells than APC are added.
16. After 16 h, the expression of our reporter eGFP reached a maximum (see ref. 14). However, depending on the particular mRNA delivered, expression times may vary and should be tested individually. Perform experiments at least in triplicate and determine the respective mean value.
17. In principle, the successful expression of your gene-of-interest can be checked via fluorescence microscopy, flow cytometry, Western Blotting or any other suitable method. Relate the number of positively transfected cells to the uptake ratio of your positive control as only APC that have taken up yeast cells can actually be transfected, e.g., if you detect your protein-of-interest in 20% of the transfected cells and your uptake control yields 50% uptake, then the ratio of positive cells is $(100/50) \times 20 = 40\%$ (see also Note 14).
18. The delivery efficacy depends strongly on the nature of your gene-of-interest and varies from no detectable expression up to 50% and more "transfected" cells.
19. Be very careful in casting the PBS–blood mixture on the Ficoll in order to avoid mixture of blood and separation medium by pipetting or pouring as this step is crucial for an effective separation of cell populations.
20. Centrifugation with brake off is necessary to not disturb the gradient; be aware that this procedure relates only to this step.
21. Avoid to carry over even small amounts of separation medium/Ficoll as it tends to be cytotoxic.
22. The erythrocytes are lysed by bursting in water. However, do not wait longer than approximately 15 s before adding PBS as a prolonged time will damage your PBMC, too.
23. One buffy coat (400 ml) yields approximately $2–4 \times 10^8$ PBMC; 1 ml whole blood contains approximately 1×10^6 PBMC.

24. As an alternative to adherence, monocytes can also be isolated from PBMC via CD14-specific antibodies coupled to magnetic beads which increases both yield and purity of the isolation. However, the isolation by adherence to plastic usually yields nice results and is not as expensive.
25. During differentiation from monocytes to DC, media must be supplemented with LPS-free FCS in order to avoid pre-maturation of the DC which would strongly decrease their capacity to engulf yeast cells.
26. Be aware that the maturation from a monocyte to a DC phenotype causes detachment of the cells as well as morphological changes.
27. Titration of the antibodies is important to minimize background, optimize signal, and save cost-extensive antibody. The optimal concentration (yielding a clearly positive signal and low background) depends strongly on your FACS device and the experimental conditions; use the concentration mentioned in the package insert of the respective antibody as starting point and perform twofold serial dilutions.
28. Use cells directly for FACS analysis or store stained cells at the most for 1 week at 4°C in the dark before performing flow cytometry. Expression of the monocyte marker CD14 should be low and the DC-specific expression marker CD1a should be high. In contrast to mature DC, immature DC show a low expression of HLA-DR, HLA-I, CD80, CD86, and CD83; see also Note 29 and Table 1.
29. Test at least four independent donors and state the respective median values as DC from different donors can differ significantly in the expression of the delivered mRNA (see Fig. 3b). As a control for DC maturation, a fraction of the yeast-loaded DC should be checked by flow cytometry for increased expression of HLA-DR, HLA-I, CD80, CD86, and CD83 (see also Table 1). Again, relate the number of positively transfected cells to the uptake ratio of your positive control as only APC that have taken up yeast cells can actually be transfected, e.g., if you detect your protein-of-interest in 20% of the transfected cells and your uptake control yields 50% uptake, then the ratio of positive cells is $(100/50) \times 20 = 40\%$ (see also Note 17).
30. After removal of monocytes from the PBMC by adhesion, the fraction of non-adherent cells represents the “peripheral blood lymphocytes” (PBL) containing also the T cells that are stimulated in the next step.
31. In our experience, having compared different plastics, the assay works best with polypropylene tubes.

Table 1
Surface marker and their expression on DCs with different maturation states

	Expression	
Surface marker	iDC	mDC
CD1a	+++	+++
CD14	−/+	−
CD80	+	++
CD86	+/−	+++
CD83	−/+	+++
HLA-DR	++	+++++
HLA-I	++	++++

iDC immature DCs, *mDC* mature DCs

32. As an alternative, you can perform the assay directly in polypropylene FACS tubes.
33. The loose cap allows contact of the cells and the medium with the CO_2-containing atmosphere.
34. Brefeldin A is added to prevent the marker cytokine IFNγ from being secreted allowing intracellular staining of this cytokine.
35. Fixation prevents proteins inside or on the surface of the cell from being degraded by proteases allowing long term storage of the cells. Further, it allows staining of intracellular cytokines that requires permeabilization of the cell membrane. Note that in Subheading 3.4.4 only receptors at the cell surface are stained and, thus, no fixation step is needed before staining.
36. The addition of FACS buffer stops the fixation procedure.
37. Before staining, fixed cells can be stored up to 48 h at 4°C. Store stained cells at the most for 3 days before performing flow cytometry.
38. As an alternative to the given protocol for cell permeabilization and fixation you can also use the “Intrasure Kit” [Cat number 641776, Becton Dickinson].
39. Saponin is needed to form pores within the cytoplasmic membrane of the cells in order to allow the entry of the antibody for intracellular staining. Prolonged incubation times will damage your cells.

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Part II

Modulation and Monitoring of Cell Metabolism

Chapter 12

Nonviral RNA Transfection to Transiently Modify T Cells with Chimeric Antigen Receptors for Adoptive Therapy

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Abstract

Redirecting T cells with a chimeric antigen receptor (CAR) of predefined specificity showed remarkable efficacy in the adoptive therapy trials of malignant diseases. The CAR consists of a single chain fragment of variable region (scFv) antibody targeting domain covalently linked to the CD3ζ signalling domain of the T cell receptor complex to mediate T cell activation upon antigen engagement. By using an antibody-derived targeting domain a CAR can potentially redirect T cells towards any target expressed on the cell surface as long as a binding domain is available. Antibody-mediated targeting moreover circumvents MHC restriction of the targeted antigen, thereby broadening the potential of applicability of adoptive T cell therapy. While T cells were so far genetically modified by viral transduction, transient modification with a CAR by RNA transfection gained increasing interest during the last years. This chapter focuses on methods to modify human T cells from peripheral blood with a CAR by electroporation of in vitro transcribed RNA and to test modified T cells for function for use in adoptive immunotherapy.

Key words: Chimeric antigen receptor, T cell, RNA, In vitro transcription, Electroporation, Adoptive cell therapy

1. Introduction

Over two decades ago, Eshhar and colleagues first described the concept of engineering T cells with an antibody-based chimeric antigen receptor (CAR) to redirect effector T cell functions towards predefined targets for use in the adoptive immunotherapy of malignant diseases (1). Since then, the field has moved at pace and first clinical trials showed remarkable efficacy (2, 3). The prototype CAR consists of its extracellular part of a protein targeting domain,

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usually a single-chain fragment of variable regions (scFv) antibody linked in frame with an IgG1 Fc spacer and of its intracellular part of a T cell receptor derived intracellular signalling domain, mostly CD3ζ (reviewed in refs. 4–6). The use of an antibody-derived binding domain circumvents the MHC restriction in T cell recognition. Consequently, not only a wide array of proteins but any antibody-recognizable antigen, for instance, carbohydrate or glycolipid moieties, are potentially targetable with an appropriate binding moiety (7, 8). Second and third generation CARs additionally incorporate co-stimulatory domains which are intended to improve and prolong T cell activation thereby enhancing antitumor activity (reviewed in ref. 5).

The CAR engineering strategies routinely use retro- or lentiviral transduction or plasmid DNA transfection of effector T cells, which results in stable genomic integration of the transgene. This allows for constitutive expression of the transgenic CAR in T cells for at least several months. However, there are several limitations and safety concerns for most currently used gene-modification approaches. The integration of the exogenous vector DNA bears the risk of irreversible genomic alterations and, at least theoretically, malignant transformation and outgrowth of leukemic T cell clones. The permanent genetic modification of T cells can create a permanent burden of potentially auto-reactive T cells due to reactivity of the transgenic CAR to physiologically expressed antigen as observed in a clinical trial (9). There has, moreover, been a growing concern that the mitogenic activation of T cells to facilitate retroviral gene transfer may have deleterious effects upon the in vivo function of the generated T cell populations (10–12). Consequently, T cell modification without the need of mitogenic pre-stimulation will be preferred.

A solution for these and other insufficiencies is the transient modification of T cells by transfection of in vitro transcribed, poly(A) mRNA encoding the respective CAR (13–16). Nontoxic square-wave electroporation is routinely used to load mRNA into the cytoplasm of the recipient cells. After mRNA transfer >80% of T cells were viable and >90% of them expressed the CAR. $CD4^+$ and $CD8^+$ T cells, as well as NK cells, can be reprogrammed by mRNA transfer. RNA transfer produces a dose-dependent expression, allowing the control of the level of CAR expression over a wide range. RNA transfer, moreover, allows simultaneous co-expression of two or more different RNA transcripts (17, 18). Increasing the dose of mRNA over an optimal concentration, however, increased activation-induced cell death. Compared to viral transduction, the RNA based reprogramming procedure is fast; in particular, the transgenic CAR is expressed within 12 h after RNA electroporation. Pre-activation of T cells is not required; mRNA can be electroporated in completely unstimulated T cells (13).

Unlike integrating DNA or viral particles, mRNA persistence and thereby CAR expression is transient, declines to half-maximal levels within 2 days and is undetectable after 1 week (15). Due to the incapability of transferred mRNA to replicate and to integrate into the host genome, there are no integration-associated safety concerns. If unintended auto-reactive T cells are generated, the auto-aggressive pathology will be temporary due to the short half-life of CAR expression, without the need of therapeutic T cell elimination in long-term. Acute, however, temporary toxicity may occur.

Redirected activation of CAR T cells modified by RNA was thoroughly evaluated in a site-by-site comparison with retrovirus modified T cells (15). RNA engineering was at least as efficient as retroviral gene transfer; human T cells reprogrammed by RNA transfer are fully activated by CAR engagement of antigen, resulting in cytokine production and tumor cell lysis as are retrovirally manufactured T cells. With respect to tumor eradication, multiple injections of RNA modified T cells expressing a tumor recognizing CAR can mediate regression of an autologous disseminated tumor in the preclinical models (16, 19).

Since RNA modification of T cells avoids some major disadvantages of permanent genetic engineering, the strategy is gaining increasing interest and is currently translated into clinical practice. Protocols for ex vivo manufacturing of mRNA (17), an optimized vector backbone for in vitro RNA transcription (19), a procedure for high throughput electroporation of up to 10^8 primary T cells (20) and for generating medium-scale batches of CAR-expressing T cells were recently reported (21). The protocols are currently scaled up to adapt them to Good Manufacturing Practice (GMP) conform production of CAR T cells for clinical applications. Taken together the approach may increase the therapeutic index of CAR engineered T cells without the associated safety concerns of integrating vectors.

We here provide a detailed laboratory protocol for mRNA production that involves in vitro transcription of plasmid DNA templates followed by poly(A) addition, for engineering human peripheral blood T cells with in vitro-transcribed RNA and for testing for CAR-mediated T cell activation.

2. Materials

Prepare all reagents in a sterile environment at room temperature.

2.1. Nonviral Vectors

RNA transcription vector (see Note 1), which was isolated using a standard plasmid DNA purification kit (Qiagen, Hilden, Germany).

2.2. Human T Cells

The following sources of human T cells are suitable for electroporation with CAR RNA.

1. Peripheral blood mononuclear cells (PBMC) isolated from blood donations or buffy coats using standard Ficoll-density gradient centrifugation methods. Removal of dead cells prior to use by Ficoll density centrifugation or other dead cell removal methods (e.g., Miltenyi Biotec "dead cell removal kit") is strongly recommended to avoid reduction in transfection efficiency.
2. Isolated T cells or T cell subsets by using bead-based systems provided by Miltenyi Biotec or Dynal-Invitrogen or by flow cytometry based cell sorting.

2.3. Activation of T Cells

1. Ficoll-Paque™ solution: 5.7% (w/v) "Ficoll®-400" and 9% (w/v) sodium diatrizoate ("Ficoll-Paque PLUS", Amersham Pharmacia Biotech, Uppsala, Sweden).
2. T cell medium: RPMI 1640, 10% (v/v) heat-inactivated fetal calf serum (FCS), 25 mM Hepes, 50 nM 2-mercaptoethanol, 2 mM L-glutamine, penicillin (100 IU/mL), streptomycin (100 μg/mL) (medium and supplements from Lonza/Gibco) (see Note 2).
3. Agonistic anti-CD3 antibody (OKT3), 50 μL aliquots of 300 μg/mL stock solutions prepared in phosphate buffered saline (PBS), 2% (w/v) albumin and stored at −80°C. Working solution (30 μg/mL) generated by diluting one 50 μL aliquot with 450 μL of T cell medium and stored at 4°C (see Note 3).
4. Recombinant Human IL-2 (Chiron/Novartis Pharma GmbH, Nürnberg, Germany). 400 μL aliquots of 10^6 IU/mL prepared from the lyophilized powder in PBS (see Note 4 and stored at −80°C). Working stock (10^5 IU/mL) generated by diluting one 400 μL aliquot with 3.6 mL of T cell medium and stored at 4°C. Beads "MACSi beads" (Miltenyi Biotec, Bergisch Gladbach, Germany); "Human T cell activator beads" (Dynal-Invitrogen, Grand Island, NY)

2.4. In Vitro Transcription of CAR RNA

1. E-PAP buffer (fivefold, composition not declared), 100 μL $MnCl_2$ (25 mM), 100 μL ATP solution (10 mM), E-PAP enzyme mix (2 U/μl), all solutions are components of the "mMESSAGE mMACHINE T7 Ultra Kit" (Applied Biosystems/Ambion).
2. RW1 and RPE buffer (QIAGEN "RNeasy Mini Kit", compositions are not declared); "RNeasy mini-column" (QIAGEN).
3. 10× FA gel buffer: 200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0; FA running buffer: 1× FA gel buffer, 2.5 M formaldehyde; 5× RNA loading buffer: 0.25% (w/v) bromophenol blue, 4 mM EDTA, 0.9 M formaldehyde, 20%

(v/v) glycerol, 30.1% (v/v) formamide; 4× FA gel buffer (stable for 3 months at 2–8°C); 37% (12.3 M) formaldehyde; ethidium bromide stock solution (10 mg/mL).

2.5. RNA Electroporation of Human T Cells

1. OptiMEM and RPMI 1640 growth medium without serum and phenol red (GIBCO/Invitrogen, Darmstadt, Germany).
2. mMESSAGE mMACHINE T7 Ultra kit (Applied Biosystems/Ambion, Austin, TX, USA).
3. 4-mm electroporation cuvette (Bio-Rad, Munich, Germany).
4. "Genepulser XCell" Aparatus (Bio-Rad, Munich, Germany).
5. MLPC medium: RPMI 1640 (GIBCO/Invitrogen, Darmstadt, Germany) supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 20 mg/L gentamycin, 10 mM HEPES, 1 mM sodium pyruvate, 1% MEM nonessential amino acids, 20 U/mL IL-7 (ImmunoTools, Friesoythe, Germany).
6. "RNeasy mini kit" (QIAGEN, Hilden, Germany).
7. Centrifuge with cooling and operation at >10,000 × *g*.
8. Electroporation device Gene Pulser Xcell (Bio-Rad, Hercules, CA).

2.6. Flow Cytometric Analysis of CAR Expressing T Cells

1. Staining buffer for immunofluorescence: PBS containing 0.1% (v/v) NaN_3 (optional plus 1% [w/v] bovine serum albumin).
2. $F(ab)_2$ goat-anti-human IgG1-FITC, 1:100 diluted in PBS (Southern Biotechnology, Malvern, PA).
3. Propidium iodide stock solution, 1 mg/mL in ddH_2O.
4. Cytofluorometer (e.g., FACS Canto™ cytofluorometer equipped with the Diva™ analysis software, Becton Dickinson, Mountain View, CA).

2.7. Functional Analysis of T Cells: CAR Mediated T Cell Activation

1. Solution of 10 μg/mL of an anti-human IgG1 Fc antibody and, for control, of an anti-mouse IgG1 antibody (Southern Biotechnology, Birmingham, AB, USA) in coating buffer, filtered through a 0.2 μm filter.
2. Coating buffer for plastic-immobilization of antibodies: Mix 0.8 mL of 0.2 M $NaHCO_3$ solution (stock A) with 1.7 mL of 0.2 M Na_2CO_3 solution (stock B) and add ddH_2O to a final volume of 10 mL (pH 9.8). If required, scale up to a higher volume.
3. ELISA plates, e.g., PolySorp™ and MaxiSorp™ (both Nunc, Roskilde, Denmark).
4. Washing buffer for use in ELISA: PBS, 1% (v/v) Tween® 20 (Merck, Darmstadt, Germany).
5. Blocking buffer for use in ELISA: 3% (w/v) bovine serum albumin (BSA) in PBS, 1% (v/v) Tween® 20.

6. Human IFN-γ ELISA reagents: anti-human IFN-γ capture mAb, biotinylated anti-human IFN-γ mAb, recombinant IFN-γ standard (all Endogen, Woburn, MA, USA). Prepare a dilution of 1 μg/mL mouse anti-human IFN-γ antibody in coating buffer. Prepare a dilution of recombinant human IFN-γ standard (Endogen, Woburn, MA, USA) with serial dilutions of 20 ng/mL IFN-γ in RPMI 1640 medium, 10% (v/v) FCS. Prepare a dilution of a biotin-conjugated anti-human IFN-γ detection antibody (Endogen, Woburn, MA, USA) to a final concentration of 0.5 μg/mL.
7. Peroxidase-labeled streptavidin solution, 1:10,000 dilution in PBS (Roche Diagnostics, Mannheim, Germany); peroxidase substrate solution, 1 mg/mL ABTS® (peroxidase substrate) in ABTS®-substrate buffer (Roche Diagnostics, Mannheim, Germany).
8. Respiring cells indicator: XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5(phenyl-amino)carbonyl-2H-tetrazoliumhydroxide), a substrate for mitochondrial dehydrogenases (1 mg/mL) ("Cell Proliferation Kit II", Roche Diagnostics, Mannheim, Germany).
9. ELISA reader (e.g., MWG Biotech GmbH, Ebersberg, Germany).

3. Methods

Carry out all procedures at room temperature in a Class II microbiological safety cabinet unless otherwise stated.

3.1. Isolation of Human T Cells from Peripheral Blood

1. Dilute heparinised peripheral blood or alternatively the content of a buffy coat 1:1 (v/v) with PBS.
2. Pipet 15 mL of Ficoll-Paque solution into a 50 mL-conical tube and overlay the Ficoll with 30 mL diluted blood.
3. Spin the density gradient at 800 × *g* for 30 min at room temperature (RT) (brakes switched off!). The mononuclear cells will be concentrated in the interface between plasma and Ficoll-Paque solution.
4. Remove most of the plasma and collect the mononuclear cells carefully from the interface and wash the cells three times in PBS (see Note 5).
5. Resuspend the cells at a density of $1–2 \times 10^6$ cells/mL in T cell growth medium supplemented with 200 U/mL IL-2, and 100 ng/mL anti-CD3 antibody (OKT3).

6. Culture the cells for 2 days at 37°C in a humidified atmosphere with 5% CO_2.
7. Collect the cells, wash twice with complete medium and resuspend in T cell medium with 200 U/mL IL-2.

3.2. Activation of Human T Cells Prior to RNA Modification

T cells can but do not need to be activated prior to RNA modification.

1. Plate PBMC at $1–2 \times 10^6$ cells per mL of human T cell medium in a suitable size culture vessel (see Note 6).
2. Add 1 μL of agonistic anti-CD3 mAb per mL of culture (final concentration 30 ng/mL, see Note 3).

Alternative protocol 1

1. Coat non-tissue culture wells or flasks with anti-CD3 mAb (1 μg/mL in PBS, see Note 3) for 1 h at 37°C, 2–4 h at room temperature (RT) or overnight at 4°C.
2. Aspirate mAb and block wells or flask with T cell medium for 20 min at RT.
3. Adjust T cells to a concentration of 1×10^6 cells/mL in T cell medium.
4. Aspirate T cell medium from the wells.
5. Plate T cells onto mAb coated plate. Add 1 μL of IL-2 per mL of culture to achieve a final concentration of 100 IU/mL (see Note 7).

Alternative protocol 2

1. Prepare beads as described by the manufacturer (see Note 8).
2. Plate T cells at $1–2 \times 10^6$ cells per mL of T cell medium in a suitable size culture vessel as indicated by the manufacturer and add beads as indicated by the manufacturer.
3. Add 1 μL of IL-2 per mL of culture to achieve a final concentration of 100 IU/mL.

3.3. Human T Cell Expansion

Human T cells continue to expand at a rapid rate for the first 10–12 days after activation and then begin to plateau. During this time, T cell growth should be assessed every 2–3 days and culture should be readjusted to a cell concentration of 0.5×10^6 cells/mL by the addition of fresh T cell media to the culture. Add fresh IL-2 to achieve the final concentration of 100 IU/mL.

3.4. In Vitro Transcription of CAR RNA

1. Linearize the vector DNA, e.g., pGEM4Z (22), by restriction with a restriction enzyme downstream of the insert to be transcribed, e.g., *SpeI* for pGEM4-Z/A64.
2. Analyze a sample of linearized template DNA by agarose gel electrophoresis to confirm that cleavage is complete.

3. Purify DNA by phenol–chloroform extraction.
4. Precipitate DNA with EtOH.
5. In vitro transcribe RNA using the "mMESSAGE mMACHINE T7 Ultra kit" designed for the in vitro synthesis of large amounts of efficiently and correctly capped RNA. This kit also includes reagents for in vitro poly(A) tailing ("E-PAP") to make mRNA for subsequent transfections. For RNA transcription in a 200 μg scale, the following reaction mixture is recommended: 10 μg plasmid DNA, 100 μL 2×NTP/CAP (15 mM ATP, 15 mM CTP, 15 mM UTP, 3 mM GTP, 12 mM ARCA), 20 μL T7 polymerase running buffer (tenfold; salts, buffer, dithiothreitol and other ingredients), 20 μL T7 polymerase enzyme mix, H_2O ad 200 μL (see Note 9).
6. Incubate 3 h at 37°C.
7. Add 10 μL Turbo-DNase I (component of the mMESSAGE mMACHINE T7 Ultra kit), incubate 1.5 h at 37°C.
8. For poly(A) tailing of 200 μg transcribed RNA in 200 μL, add the following reagents in this order: 360 μL H_2O, 200 μL 5-fold E-PAP buffer, 100 μL $MnCl_2$ (25 mM), 100 μL ATP solution (10 mM), mix.
9. Add 40 μL E-PAP enzyme mix, mix.
10. Incubate at 37°C for 1 h.
11. Stop incubation on ice, continue with next step or freeze RNA at –80°C (see Note 10).
12. Transfer RNA solution in 15 mL polypropylene tube.
13. Add 1.5 mL RLT buffer, 15 μL β-mercaptoethanol, 1.1 mL EtOH, mix thoroughly.
14. To purify transcribed RNA, load up to 700 μL RNA solution per "RNeasy mini-column", 3 columns are needed for 200 μl transcription assay (see Note 11).
15. Centrifuge 15 s at ≥8,000 × *g*, RT, discard flow through.
16. Add 700 μL RW1 buffer each and centrifuge 15 s at ≥8,000 × *g*, RT, discard flow through.
17. Insert columns into 2 mL collection tubes.
18. Add 500 μL RPE buffer, centrifuge 15 s at ≥8,000 × *g* at RT, discard flow through.
19. Add 500 μl RPE buffer, centrifuge 2 min at ≥8,000 × *g*, RT, discard flow through.
20. Insert columns into new 2 mL collection tubes.
21. Centrifuge 2 min at ≥8,000 × *g*, RT.
22. Insert columns into RNase-free 1.5 mL Eppendorf tubes.
23. Add 40 μL nuclease-free H_2O, incubate 1 min at RT.

24. For RNA elution, centrifuge 2 min at ≥8,000 × *g*, RT.
25. Place RNA on ice for immediate use or freeze at −80°C.
26. Monitor integrity of RNA by formaldehyde agarose (FA) gel electrophoresis (0.8–1.2% (w/v) agarose gels). Prepare gels by adding 0.8–1.2% (w/v) agarose to 1× FA gel buffer and heat mix in a microwave or boiling water bath until agarose is dissolved. Add nuclease-free H_2O if volume considerably reduced during heating due to evaporation. Cool the agarose to 65–70°C. Add formaldehyde to a final concentration of 2.5 M and add 1 μL of ethidium bromide stock solution. Pour the agarose solution onto the gel tray in a fume hood and load samples on solid gel.

3.5. RNA Electroporation of T Cells

1. Centrifuge T cells at 200 × *g*, 10 min, RT, resuspend cells in 10–50 mL RPMI 1640 without serum and phenol red.
2. Centrifuge T cells at 200 × *g*, 10 min, RT, resuspend in 10–50 mL OptiMEM.
3. Centrifuge T cells at 200 × *g*, 10 min, RT, resuspend in a density up to 120×10^6 cells per mL OptiMEM, minimum volume 100 μL.
4. Pipet 15 μg RNA into an electroporation cuvette.
5. Add 100 μL cell suspension, cell concentration should be below 120×10^6 cells per mL, mix by pipetting, avoid bubble formation.
6. Immediately place cuvette into the "Gene Pulser Xcell" electroporation machine.
7. Pulse cells with a square wave pulse, 500 V, 5 ms.
8. Immediately transfer cells to MLPC medium and incubate at 37°C for 4–6 h.
9. The number of CAR modified T cells and the functional activity of the CAR T cells can be assessed 1 day after electroporation. Determine CAR expression by flow cytometry as described in subheading 3.6.
10. Transfection efficiency is routinely >90%. If though enrichment for CAR T cells is required, we recommend immuno-selection methods, e.g., Fluorescence Activated Cell Sorting (FACS) or Magnetic Activated Cell Sorting (MACS), to either bind the CAR directly or to capture engineered T cells based upon expression of a marker gene co-expressed within the vector, e.g., truncated CD34, truncated LNGFR, eGFP or smaller tags.

3.6. Flow Cytometric Analysis of CAR Expressing T Cells

1. Pipet transfected T cells (approximately 2×10^5 cells) into a 4-mL-polypropylen test tube.

2. Centrifuge the cell suspension for 3 min at 400 × *g*, discard supernatant by inversion of the tube, and wash the cells once in ice-cold PBS.
3. Discard the washing solution and resuspend the cells in 100 μL of staining buffer containing an appropriate dilution of a FITC-conjugated anti-human IgG1 Fc antibody. Vortex test tubes prior to incubation.
4. Incubate 30 min on ice.
5. Wash the cells twice with ice-cold PBS.
6. Discard the washing solution, resuspend the cells in 100 μL of staining buffer containing 1 μg/mL propidium iodide (1:1,000 dilution of a 1 mg/mL stock solution in ddH_2O, Sigma, St. Louis, MO). Analyze cells by flow cytometry. To exclude dead cells, set appropriate life gates prior to analysis (see Note 12).

3.7. Functional Analysis of T Cells: CAR Mediated T Cell Activation

Harvest T-cells from culture vessels, wash, count, and resuspend in suitable assay culture media and dilute cells to a concentration needed for specific assays.

3.7.1. CAR Cross-linking by an Anti-IgG1 Antibody

1. Coat the wells of an ELISA plate with the diluted anti-human IgG1 Fc antibody and for control with the anti-mouse IgG1 antibody (50 μL/well) and incubate overnight at 4°C.
2. Aspirate the antibody solution and wash the plate three times with 200 μL/well sterile PBS.
3. Wash transfected T cells three times with RPMI 1640 medium to remove residual IL-2 and resuspend 0.5–1×10^6 cells per mL in RPMI 1640 medium, 10% FCS.
4. Add 1–2×10^5 T cells in 200 μL per well to the coated microtiter plate and incubate for 48 h at 37°C.
5. Analyze 100 μL of cell free supernatant per well for IFN-γ as described below.

3.7.2. CAR Engaging Target Cells with Cognate Antigen

Specific activation of CAR engineered T cells can be monitored by recording of IFN-γ secreted into the culture supernatant of engineered T cells co-cultured with allogeneic or autologous tumor cells which express the CAR recognized antigen.

1. Wash RNA modified T cells and, for control, non-modified T cells three times with RPMI 1640 medium to remove residual IL-2 and resuspend 2×10^6 cells per mL RPMI 1640 medium, 10% (v/v) FCS.
2. Suspend target cells, which express CAR recognized antigen, in RPMI 1640 medium, 10% (v/v) FCS to obtain 5×10^5 cells/mL.
3. Prepare serial dilutions of the effector T cells in 96-round-bottom-microtest-plates starting from 2×10^5 cells in 100 μL medium/well.

4. Add a 100-μL-suspension of target cells (i.e., 5×10^4 cells/well) to effector cells and incubate for 48 h at 37°C.
5. Remove 100 μL/well of the supernatant and analyze for IFN-γ as described below.

3.8. Functional Analysis of T Cells: IFN-γ ELISA

1. Coat the wells of an ELISA plate with the anti-human IFN-γ antibody (capture antibody) (100 μL/well) diluted in coating buffer and incubate overnight at 4°C.
2. Discard the antibody coating solution, wash plate three times with washing buffer (3×200 μL/well) and incubate for at least 2 h at room temperature with blocking buffer (200 μL/well).
3. Wash once with washing buffer (200 μL/well).
4. Add 100 μL of cell culture supernatant and of a dilution of a human IFN-γ standard, respectively, per well. Optional apply dilutions of the cell culture supernatant in culture medium. Incubate for 2 h at room temperature.
5. Discard the supernatants and wash five times with washing buffer (200 μL/well).
6. Add 100 μl of a biotin-conjugated anti-human IFN-γ detection antibody and incubate for 1 h at room temperature.
7. Repeat step 5.
8. Add 100 μl of peroxidase-labeled streptavidin solution and incubate for 30 min at RT.
9. Repeat step 5.
10. Add 100 μl peroxidase substrate solution per well to the test plate. Incubate for at least 30 min. Record O.D. photometrically at 405 nm utilizing an ELISA reader. A positive reaction is indicated by green color.

3.9. Cytotoxicity Assay

We here describe a procedure for a nonradioactive cytotoxicity assay based on the metabolization of XTT as described by Jost et al. (23).

1. Incubate CAR engineered T cells and mock-engineered T cells (1×10^4 cells per well) together with tumor cells in triplicates in round bottom microtiter plates for 48 h at 37°C. Control cultures are the same number of (a) T cells only (b) tumor cells only, (c) culture medium only.
2. Add XTT reagent and incubate for 90 min at 37°C.
3. Record optical density (OD) colorimetrically at an absorbance wavelength of 450 nm and a reference wavelength of 650 nm. Altered OD indicates reduction of XTT to formazan by viable tumor cells.
4. Calculate cytotoxicity of T cells as follows: cytotoxicity (%) = [1 − OD (experimental wells − corresponding number of effector cells)/OD (tumor cells without effector cells − medium)] × 100.

4. Notes

1. Authors obtained good experience with the pGEM4Z vector (kindly provided by Kris Thielemans) having inserted the CAR cDNA. For in vitro transcriptions, the vector DNA was linearized, purified by phenol–chloroform extraction and ethanol precipitation, and used as DNA templates.
2. Hepes and bicarbonate-buffered RPMI 1640 medium is available through various suppliers; alternatively, one can use RPMI 1640 based synthetic and serum-free media for human T cell cultures, e.g., AIMV (Invitrogen), and XVivo 15 (Lonza). These serum-free media can be supplemented with 2–5% human serum or plasma or blended with RPMI 1640 (24, 25), however, we do not recommend human serum or plasma since it prevents good staining for CAR expression. Batches of FCS can have widely varying effects upon T cell growth. Batch testing of serum using the readout of degree of expansion of mitogen-activated T cells over a set period of time is recommended to identify optimal batches of fetal bovine serum for T cell culture. Likewise, human serum batches may vary and it is advised to pool and test serum from different donors before use.
3. Additional antibodies may be included, e.g., anti-CD28, which can aid co-stimulation of T cells. At present, there is little published evidence to determine that the inclusion of additional mAbs in this setting improves the activation process, but preliminary data suggest that alternative activating mAbs can modulate the phenotype of the expanded CAR T cell population. Hence, empirical testing is advised to determine the advantage of including additional antibodies. For coating purpose prepare stocks and working solutions of mAbs in PBS.
4. Alternatively, lyophilized IL-2 (18 M IU, Chiron) can be reconstituted with 1.2 mL sterile water and subsequently diluted in 5% glucose supplemented with 2% albumin.
5. We routinely depleted the number of monocytes by plastic adherence as follows: Ficoll-isolated mononuclear cells were resuspended in RPMI 1640 medium without serum (about 5×10^6 cells/mL) and incubated in 150 mm tissue culture plates for 1 h at 37°C. Non-adherent cells were collected, counted and activated as described above.
6. Maintaining a large surface area to facilitate optimal gas exchange is important to achieve maximal T-cell viability. General guidelines are as follows: $\leq 0.5 \times 10^6$ per cm^2, which

equals ≤1 mL per well of a 24-well plate; ≤4.5 mL per well of a 6-well plate; <12.5 mL for a T25 flask; <37.5 mL for a T75 flask; and <87.5 mL for a T175 flask.

7. Aim at achieving a functional level of IL-2 that equates to 100–360 IU per mL. Higher levels of IL-2 can drive higher levels of lymphocyte-activated killer (LAK) activity potentially resulting in enhanced background activity when assaying CAR function. In fact, during human T cell activation (not expansion) exogenous IL-2 is redundant and may be omitted. Notably, mouse T cells may expand better in the presence of IL-15 (Peprotech: 50 ng per mL) when compared to IL-2. Alternative common-γ cytokines such as IL-7, IL-15, and IL-21 are being actively investigated and can modulate the phenotype of the expanded CAR T cell population (*see* amongst others, Pouw et al. (26)).
8. Other artificial antigen presenting cells (aAPC's) which incorporate antibody-based activation using a cell-based system are also being developed (27).
9. All equipments and reagents must be RNase-free. Most equipments and salt solutions can be autoclaved for 2 h.
10. For control of RNA integrity, 5 μL of this solution can be run in a denaturing gel electrophoresis.
11. Binding capacity of "RNeasy mini-columns" is about 100 μg RNA.
12. We identified transfected T cells by double immunofluorescence by simultaneous incubation with a phycoerythrin (PE)-conjugated anti-CD3 antibody (e.g., Dako, Hamburg, Germany) and a FITC-conjugated anti-human IgG Fc antibody (e.g., Southern Biotechnology, Malvern, PA). CARs without an extracellular spacer domain can be detected by reagents with specificity for the scFv antigen-binding domain, e.g., an anti-idiotypic antibody. Alternatively, small tags like myc-tag can be inserted into the extracellular part of the CAR to allow detection by the respective antibody, i.e., an anti-myc-tag antibody (Cell signaling, Boston, MA, USA). We identified transduced T cells which harbor this type of CAR by simultaneous staining with 10 μg/mL of a scFv-specific anti-idiotypic mAb (IgG1) and 5 μg/mL of the anti-CD3 mAb OKT3 (IgG2a). Bound antibodies were detected by a FITC-conjugated $F(ab)'_2$ goat-anti-mouse IgG1 and a PE-conjugated $F(ab)'_2$ goat-anti-mouse IgG2a antibody, respectively (each diluted 1:1,000, Southern Biotechnology, Birmingham, AB, USA).

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Chapter 13

Natural Killer Cell Reprogramming with Chimeric Immune Receptors

Noriko Shimasaki and Dario Campana

Abstract

Natural killer (NK) cells are emerging as a new tool for cell therapy of cancer. However, some cancer subtypes are relatively resistant to NK cell cytotoxicity. Expression of anti-CD19 chimeric signaling receptors can enhance NK-cell reactivity against CD19+ leukemia and lymphoma cells. Here we describe a method to enforce expression of such receptors in human NK cells relying on electroporation of mRNA and compare it to retroviral transduction of cDNA. These methods are applicable to the reprogramming of NK cells with chimeric receptors specific for other antigens expressed on cancer cells as well as with molecules that can modulate NK cell function.

Key words: Natural killer cells, Cell therapy, Retroviral transduction, Electroporation, Chimeric receptors, Acute lymphoblastic leukemia, Non-Hodgkin lymphoma

1. Introduction

Natural killer (NK) cells can identify virally infected or transformed cells through a panel of cell surface receptors which interact with their corresponding ligands on target cells: the resulting activating signals can trigger a rapid response that culminates in cytotoxicity (1). In addition to activating receptors, NK cells also express inhibiting receptors which suppress killing (1). An important set of such receptors is represented by killer immunoglobulin-like receptors (KIRs) which recognize major histocompatibility complex (HLA) Class I molecules. Upon binding to specific HLA Class I molecules, KIRs elicit signals that inhibit NK cytolytic activity, a mechanism that protects autologous cells from cytotoxicity (1, 2). Nevertheless, it has been reported that NK cell function correlates

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with cancer susceptibility, with individuals whose NK cells have a lower cytotoxic capacity being at a higher risk (3). This notion was recently reinforced by a study pointing to the presence of distinct isoforms of the NK-activating receptor NKp30 in healthy individuals and in patients with gastrointestinal tumors: the latter had predominant expression of the immunosuppressive NKp30c isoform which was associated with decrease NK cell function and an inferior survival rate (4). Despite these associations, clinical studies of the therapeutic effects of autologous NK cell infusions generally failed to yield very encouraging results (5).

During allogeneic hematopoietic stem cell transplantation (HSCT), a proportion of NK cells derived from donor stem cells may have the capacity to kill tumor cells of the recipient if these lack a cognate HLA Class I epitope. The presence of these "alloreactive" donor NK cells has been correlated with improved survival after HSCT in patients with acute leukemia (6–12). Notably, NK alloreactivity does not appear to cause damage to non-hematopoietic tissues which presumably lack of appropriate stimulatory ligands, and it produces no evidence of graft-versus-host disease (GvHD) (7). Allogeneic NK cells can also exert antitumor activity when infused as a purified blood product in a non-HSCT setting (13, 14). However, NK cells are a small subset (about 10%) of peripheral blood lymphocytes and to obtain them in adequate numbers for clinical application can be problematic. Interleukin (IL)-2 can stimulate proliferative responses but only a fraction of cells sustains growth (15–17). There have been reports of considerable NK cell expansions after about 3 weeks of culture in the presence of IL-2 (18) or IL-2 and OKT3 (19). In our experience, IL-2 alone or in combination does not induce a significant expansion of CD56+ CD3-NK cells (20). Most studies indicate the additional signals provided by accessory cells are necessary to induce robust expansions of these cells (16, 21–23). Accessory cells used for this purpose include monocytes (24), B-lymphoblastoid cells (25, 26), and a Wilms tumor cell line (27).

We found that coculture with the leukemia cell line K562 genetically modified to express two NK-stimulatory molecules, 4-1BB ligand (4-1BBL) and IL-15, produced vigorous NK cell proliferation and activation (20, 28, 29). 4-1BBL triggers activation signals after binding to 4-1BB (30), while IL-15 is a cytokine known to promote NK-cell development and the survival of mature NK cells (31–34). IL-15 has greater biological activity when presented on the cell membrane of stimulatory cells, rather than in its soluble form (35–37). Therefore, we made a construct containing the human *IL-15* gene fused to the gene encoding the human *CD8*α transmembrane domain, and used it to transduce K562 cells (28). NK cell expansions induced by K562-mb15-41BBL cells have proven to be very reliable. In our initial experiments with 50 donors using RPMI-1640 tissue culture medium, median NK cell expansion after 7 days of culture was 21.6-fold (range, 5.1–86.6-fold; $n = 50$); median NK cell recovery relative to input cells increase to 152-fold (70–744-fold; $n = 5$) after 14 days, and 277-fold (201–1,459-fold) after 21 days (20). Using large-scale conditions and SCGM medium, median 7-day expansion was 90.5-fold (range, 33- to 141-fold; $n = 12$) (20). In contrast to cultures with monocytes or B-lymphoblastoid cells, which also induce vigorous expansions of T lymphocytes, coculture with K562-mb15-41BBL cells does not stimulate T cell proliferation. This is an advantage in the context of infusions of allogeneic NK cells where activated lymphoid cells would present a high risk of graft-versus-host disease. K562-mb15-41BBL are lethally irradiated before culture and they are lysed by the expanding NK cells. Therefore, the risk of infusing viable K562-mb15-41BBL is very low. The method for expanding CD56$^+$ CD3$^-$ NK cells from peripheral blood with the K562-mb15-41BBL cell line is described in this chapter.

In addition to increase the number of NK cells available for infusion, methods for NK cell activation may also increase NK cell cytotoxicity. A possible explanation for this is that activated NK cells are poised to form a lytic synapse with target cells and the initiation steps that follow approximation of NK cells to target cells are not required (38). Indeed, NK cells expanded in coculture with K562-mb15-41BBL cells can exert remarkable cytotoxicity against a variety of malignancies including acute myeloid leukemia and sarcomas, and are more powerful than non-expanded NK cells (20, 39). However, both primary and expanded NK cells are relatively ineffective against other malignancies such as ALL, neuroblastoma, and osteosarcoma, as well as adult solid tumors. The reasons for these differences in sensitivity are poorly understood but it is likely to depend, at least in part, on the balance of ligands for NK inhibitory and activating receptors expressed by the tumor cells (1). In a study of childhood solid tumors, we examined whether the variable susceptibility to NK cell cytotoxicity was related to their level of HLA Class I expression, or to that of ligands known to trigger NK cell activation, including MIC A/B, ULBP1, ULBP2, and ULBP3 (NKG2D ligands), CD112 and CD155 (DNAM-1), as well as of the adhesion molecules CD54 and CD58 and of HLA-E (NKG2A/C ligand) but found no significant associations (39). Hence, level of expression of individual ligands cannot predict susceptibility to NK cell cytotoxicity.

Reprogramming NK cells with chimeric immune receptors provides a way to improve their activity against cancer subtypes which are relatively resistant to NK cell cytotoxicity. Thus, NK cells expressing an artificial ("chimeric") signaling receptor against CD19, a surface molecule widely expressed in B-cell malignancies, became highly cytotoxic to ALL cells (28). Expression of an anti-CD19 chimeric receptor, which contains the stimulatory molecules CD3ζ and 4-1BB ("anti-CD19-BBζ") could overcome KIR inhibitory

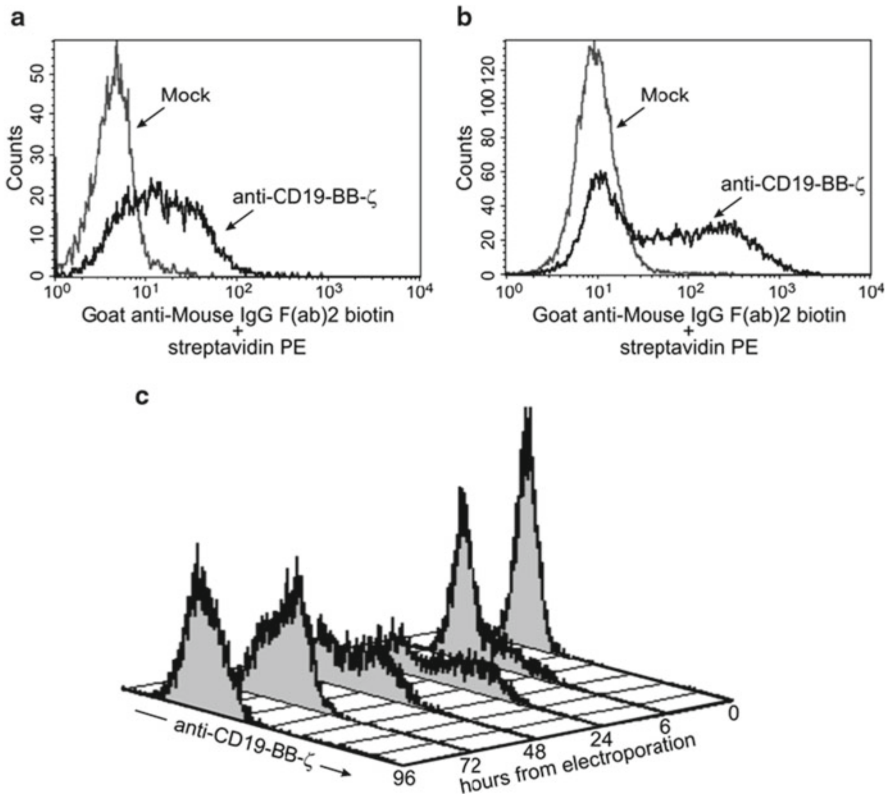

Fig. 1. Expression of anti-CD19-BB-ζ receptors in NK cells after electroporation of the corresponding mRNA. (**a**) Flow cytoemtric histograms show expression in purified, not expanded CD56+ CD3− NK cells 24 h after electroporation with or without ("mock") anti-CD19-BB-ζ mRNA. (**b**) Expression in CD56+ CD3− NK cells expanded by 7-day coculture with K562-mb15-41BBL cells 24 h after electroporation with or without anti-CD19-BB-ζ mRNA. (**c**) Expression in NK cells expanded as in (**b**) at different times after electroporation.

signals and kill autologous leukemic cells (28). Likewise, expression of anti-GD2 chimeric receptors, rendered NK cells highly cytotoxic to neuroblastoma (40).

Electroporation of mRNA has proven to be very effective for expressing chimeric receptors in immune cells (41–43). We found that electroporation of mRNA encoding anti-CD19-BBζ with the method described here resulted in a median receptor expression 24 h after electroporation of 61.3% in expanded (n = 31 donors) and 40.3% in primary NK cells (n = 18 donors) with excellent cell viability (Fig. 1a, b). Expression was detectable 6 h after electroporation, reaching maximum levels at 24–48 h (Fig. 1c) (44). Specific anti-CD19 cytotoxicity remained higher than that of control cells at 96 h.

In this chapter, we describe the methods for genetic reprogramming of NK cells using electroporation of anti-CD19-BBζ receptor mRNA; a method for retroviral transduction is also described. In principle, these methods are also applicable to the expression of other receptors which may modify NK cell function.

Methods to determine the specific cytotoxicity of reprogrammed NK cells have been described in detail elsewhere (28, 45). It should be noted that these protocols have been adapted to large-scale, cGMP compliant, conditions and have been incorporated into clinical protocols.

2. Materials

2.1. Expansion of NK Cells

2.1.1. Expansion of NK Cells by Coculture with K562-mb15-41BBL Cells

1. Peripheral blood mononuclear cells (PBMC) obtained by density-gradient separation from whole blood.
2. K562 retrovirally transduced with the genes encoding 4-1BB ligand and membrane bound IL-15 (K562-mb15-41BBL) and green fluorescence protein (GFP). For a detail description of the preparation of this cell line, see refs. (20, 28).
3. SCGM or RPMI-1640 tissue culture media with 10% fetal bovine serum (FBS) and penicillin and streptomycin.
4. Recombinant human interleukin-2 (IL-2).
5. Cs-137 irradiator.

2.1.2. T-Cell Depletion

1. Cell separation buffer: phosphate-buffered saline (PBS) pH 7.2, 0.5% BSA and 2 mM EDTA.
2. RPMI-1640 medium with 10% FBS and penicillin and streptomycin.
3. Magnetic beads conjugated to antibody against human CD3, e.g., from Miltenyi Biotec (Auburn, CA; 130-050-101) or Invitrogen (Carlsbad, CA; 111.51D).
4. Magnetic separator (Quadro MACS separator, Miltenyi Biotec) and column (LD column, Miltenyi Biotec; 130-042-901) for cell sorting (depending on magnetic bead manufacturer).

2.2. Electroporation of Anti-CD19-BBζ mRNA

2.2.1. Preparation of mRNA

1. Vector plasmid containing a gene of anti-CD19-BBζ and a T7 promoter site (28, 46).
2. Restriction enzyme (*Xba*I, Invitrogen, Carlsbad, CA) and suitable digestion buffer.
3. Transcription kit (mMESSAGE mMACHINE T7 Ultra Kit, Ambion, Austin, TX) containing T7 Enzyme Mix, T7 2X NTP/ARCA, 10× T7 Reaction Buffer, TURBO DNase, *E. coli* Poly (A) polymerase l (E-PAP), 25 mM $MnCl_2$, ATP solution, 5× E-PAP buffer, lithium chloride (LiCl) and nuclease-free water.

4. 95% and 70% Ethanol.
5. 3 M Sodium acetate.
6. TE buffer.

2.2.2. Electroporation Procedure (43) (see Note 1)

1. mRNA encoding anti-CD19-BBζ.
2. Electroporation buffer (MaxCyte, Gaithersburg, MD).
3. RPMI-1640 medium with 10% FBS.
4. IL-2.
5. Electroporator (MaxCyte).
6. Electroporation chamber (MaxCyte).

2.2.3. Antibody and Conjugates for Flow Cytometry

1. Goat anti-mouse IgG, $F(ab')_2$ fragment-specific antibody conjugated with biotin (catalog number 115-065-072) (Jackson ImmunoResearch, West Grove, PA).
2. Streptavidin conjugated to PerCP (catalog number 340130) (BD Biosciences, San Jose, CA) or

   Streptavidin conjugated to PE (catalog number 016-110-084) (Jackson ImmunoResearch).

2.3. Retroviral Transduction

2.3.1. Production of Retroviral Vector Supernatant

1. 293 T human embryonic kidney cell line (American Type Culture Collection, ATCC, Rockville, MD; CRL-11268).
2. Retroviral vector plasmid containing the anti-CD19-BBζ construct (28, 46).
3. pEQ-PAM3(-E); the amphotropic helper packaging plasmid.
4. pRDF; plasmid encoding the RD114 envelope.
5. DMEM medium.
6. DMEM medium with 10% FBS.
7. RPMI-1640 medium with 10% FBS.
8. Cationic lipid transfection agent (FuGENE 6 Transfection Reagent, Roche, IN, or equivalent).
9. Dry ice.
10. 10-cm Tissue culture dish.
11. 4.5 ml Cryotube.

2.3.2. Concentration of Retroviral Vector Supernatant

1. Precipitation solution (PEG-it Virus Precipitation Solution (5×), System Biosciences, Mountain View, CA, or equivalent).
2. RPMI-1640 medium with 10% FBS.

2.3.3. Determination of Retroviral Titer

1. HeLa cell line (ATCC; CCL-2).
2. Vector supernatant.

3. DMEM medium 10% FBS.
4. 8 mg/ml Polybrene stock solution.

2.3.4. Preparation of Fibronectin Fragment-Coated Tubes

1. Recombinant fibronectin fragment (RetroNectin, Roche, or equivalent).
2. PBS.
3. PBS with 2% bovine serum albumin (BSA).
4. Cell culture grade water.
5. 0.22 μm filter.

2.3.5. Transduction

1. RPMI-1640 medium.
2. RPMI-1640 medium with 10% FBS.
3. IL-2.fi

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3. Methods

3.1. Expansion of NK Cells

3.1.1. Expansion of NK Cells by Coculture with K562-mb15-41BBL Cells

1. Resuspend PBMC in SCGM or RPMI-1640 plus 10% FBS at a concentration of 1.5×10^6 cells/ml (see Note 2).
2. Irradiate the K562-mb15-41BBL cells at 100 Gy γ-irradiation (see Note 3).
3. Wash the irradiated K562-mb-41BBL cells once and resuspend in RPMI-1640/10% FBS medium at a concentration of 1.0×10^6 cells/ml.
4. Mix the same volume of the resuspended PBMC and K562-mb15-41BBL cells in a conical tube and add 10 IU/ml IL2.
5. Transfer the mixed cells to a tissue culture plate, flask, or bag (see Note 4).
6. Incubate at 37°C in 5% CO_2.
7. On days 2, 4, and 6, remove half of the supernatant gently and add the same volume of RPMI-1640/10% FBS medium with 20 IU/ml IL2 (see Note 5).
8. On day 7, count the cells (see Notes 6–8).

3.1.2. T-Cell Depletion

1. Collect the expanded cells after 7 days of culture with K562-mb15-41BBL cells in a conical tube and determine the cell number.
2. Centrifuge at $400 \times g$ for 5 min and remove the supernatant.
3. Resuspend the cell pellet in separation buffer at a concentration of 1.25×10^8 cells/ml.

4. Add 20 µl of magnetic beads conjugated to anti-CD3 monoclonal antibody per 10^7 cells.
5. Mix well and incubate for 15 min at 4°C.
6. Wash cells at 400 × *g* for 5 min.
7. Resuspend the cells in separation buffer at a concentration of 2×10^8 cells/ml.
8. Place a proper column in a magnet field of a suitable separator and rinse the column with 2 ml of separation buffer.
9. Apply the cell suspension onto the column.
10. Collect unlabeled cells which pass through the column
11. Wash the column with 2 ml of separation buffer twice and collect total effluent.
12. Centrifuge the effluent at 400 × *g* for 5 min.
13. Resuspend the cells in RPMI-1640/10% FBS medium at a concentration of 2.5×10^6 cells/ml.
14. Add 100 IU/ml IL2.
15. Transfer the cells to a tissue culture plate, flask, or bag.
16. Incubate at 37°C in 5% CO_2.
17. Every 2 days, remove half of the supernatant gently and add the same volume of RPMI-1640/10% FBS medium with 200 IU/ml IL2 until use (see Note 9).

3.2. Electroporation of Anti-CD19-BBζ mRNA

3.2.1. Preparation of mRNA

1. Linearize the vector plasmid containing the anti-CD19-BBζ gene and a T7 promoter site with a *Xba*I restriction enzyme downstream of the gene to be transcribed.
2. Purify the linearized plasmid by ethanol precipitation with 3 M sodium acetate and 95% ethanol.
3. Dissolve the purified plasmid in TE buffer at a concentration of 0.5–1 mg/ml (see Note 10).
4. To transcribe the gene and produce a cap structure, mix 0.1–1 µg of the dissolved plasmid, 2 µl of T7 Enzyme mix, 10 µl of T7 2× NTP/ARCA, 2 µl of 10x T7 reaction buffer and nuclease-free water to total 20 µl in a RNase-free tube (see Notes 11 and 12).
5. Incubate at 37°C for 2 h.
6. Add 1 microliter of TURBO DNase.
7. Incubate at 37°C for 15 min.
8. To add poly (A) tails to the capped RNA, add 10 µl of 25 mM $MnCl_2$, 10 µl of ATP solution, 20 µl of E-PAP buffer, and 36 µl of nuclease-free water and mix well.
9. Add 4 microliter of E-PAP and mix.

10. Incubate at 37°C for 60 min.
11. To purify the RNA, add 50 μl of LiCl and mix well.
12. Incubate at −20°C for 2 h.
13. Centrifuge at 12,000 × *g* for 15 min at 4°C.
14. Remove the supernatant.
15. Add 1 ml of 70% ethanol and invert the tube several times to mix.
16. Centrifuge at 12,000 × *g* for 15 min at 4°C.
17. Remove the supernatant and air-dry the pellet for 10 min (see Note 13).
18. Dissolve the mRNA in TE buffer at a concentration of 1.0 mg/ml (see Note 14).
19. Store at −80°C until use.

3.2.2. Electroporation Procedure

1. Stimulate NK cells by adding 1,000 IU/ml IL2 the day before electroporation (see Note 15).
2. On the day of electroporation, collect the stimulated NK cells into a conical tube and centrifuge at 400 × *g* for 5 min.
3. Aspirate the supernatant and resuspend the cells in electroporation buffer (see Note 16).
4. Centrifugate at 400 × *g* for 5 min.
5. Aspirate the supernatant and centrifuge at 400 × *g* for 1 more min.
6. Aspirate the residual supernatant by pipette (see Note 17).
7. Resuspend the cells in electroporation buffer at a concentration of 2.0–3.0 × 10^8 cells/ml.
8. Transfer 50 μl of the cell suspension containing 1.0–1.5 × 10^7 cells into an electroporation chamber.
9. Add mRNA to reach 100–200 μg/ml (see Note 18).
10. Place the chamber in the electroporator and shock one time using the optimized program setting (see Note 19).
11. Transfer all cells to a well of a 96-well plate.
12. Incubate for 20 min at 37°C.
13. Add 200 μl of RPMI-1640/10% FBS medium to the well slowly and transfer the cells to a flask with RPMI-1640/10% FBS medium at a concentration of 1 × 10^6 cells/ml. Repeat this procedure several times very gently until all cells are transferred.
14. Add 100 IU/ml IL2.
15. Incubate the cells at 37°C in 5% CO_2.

16. Assay for transgene expression by staining cells with biotinylated goat anti-mouse IgG, F(ab′)$_2$ fragment followed by streptavidin conjugated to PerCP or PE. Examine cell staining by flow cytometry (see Note 20).

3.3. Retroviral Transduction

3.3.1. Production of Retroviral Vector Supernatant

1. Seed 3×10^6 293 T cells with 10 ml of DMEM/10% FBS medium in a 10-cm tissue culture dish the day before transfection (see Note 21).
2. On the day of transfection, add 30 μl of cationic lipid transfection agent to 470 μl of DMEM medium in a 1.5 ml Eppendorf tube (see Note 22).
3. Tap the tube gently to mix and incubate for 5 min at room temperature.
4. Mixed 3.5 μg of vector plasmid containing the anti-CD19-BBζ construct, 3.5 μg of pEQ-PAM3(-E) and 3.0 μg of pRDF in another 1.5 ml Eppendorf tube.
5. Add the mixture of cationic lipid/DMEM from step 3 dropwise to the mixture of DNA from step 4.
6. Tap the tube gently to mix and incubate for 45 min at room temperature.
7. Add the mixture of cationic lipid/DNA solution from step 6 dropwise to 293 T cells, swirling the dish gently.
8. Incubate at 37°C in 5% CO_2.
9. After 24 h, remove medium from the dish and add 10 ml of RPMI-1640/10% FBS medium (see Note 23).
10. After 12 h, collect the vector-containing supernatant to a 15 ml conical tube.
11. Centrifuge at 1,500 × *g* for 5 min to remove cellular debris (see Note 24).
12. Collect the supernatant and aliquot into cryotubes.
13. Incubate on dry ice for 10 min, following incubate at −80°C until use.
14. Add 10 ml of RPMI-1640/10% FBS medium to the dish from step 10.
15. Repeat from step 10 to step 14 every 12 h four times (see Notes 25–27).

3.3.2. Concentration of Retroviral Vector Supernatant

1. Add 2 ml of precipitation solution to 8 ml of vector supernatant (see Note 28).
2. Incubate at 4°C overnight (at least 12 h).
3. Precipitate the vector particles by centrifuging at 1,500 × *g* for 30 min at 4°C.

4. Aspirate the supernatant (see Note 29).
5. Centrifuge at 1,500 × *g* for 5 min.
6. Aspirate the residual supernatant.
7. Resuspend the pellet with 500 μl of RPMI-1640/10% FBS medium (see Note 30).
8. Store at −80°C until use.

3.3.3. Determination of Viral Titer

1. Seed 1×10^5 HeLa cells in each well of a 6-well plate the day before infection (see Note 31).
2. On the day of infection, remove medium from the plate and add 2 ml of DMEM/10% FBS medium.
3. Add polybrene at a concentration of 8 μg/ml.
4. Incubate the cells for 4 h at 37°C in 5% CO_2.
5. Add 10 μl of vector supernatants, swirling the dish.
6. Incubate the cells for 2 days at 37°C in 5% CO_2.
7. Replace half medium and incubate the infected cells for 2 more days at 37°C in 5% CO_2.
8. Assay for the transgene expression and determine the viral titer by flow cytometric analysis (see Note 32).

3.3.4. Preparation of Fibronectin Fragment-Coated Tubes

1. Add sterilized water at a concentration of 1 mg protein/ml to a recombinant human fibronectin fragment CH-296, and dissolve thoroughly by gently swirling.
2. Filter the solution through 0.22 μm filter.
3. Aliquot to several tubes and store at −20°C until use.
4. Dilute the filtered fibronectin fragment solution with PBS at a concentration of 100 μg/ml.
5. Add 500 μl of the diluted fibronectin fragment solution into each tube (see Note 33).
6. Allow the covered tube to stand at 4°C overnight (see Note 34).
7. Remove the fibronectin fragment solution and then add 500 μl of PBS with 2% BSA into the tube for blocking (see Note 35).
8. Allow the tubes to stand at room temperature for 30 min.
9. Transfer the collected fibronectin fragment solution from step 7 to another tube.
10. Repeat steps 5–9 three times (see Note 36).

3.3.5. Transduction

1. Aspirate PBS/2% BSA in the fibronectin fragment-coated tube (see Note 37).
2. Rinse the tube by 2 ml of RPMI-1640 medium once (see Note 38).
3. Add the vector supernatant at a multiplicity of infection (MOI) of 1–5, 0.5×10^6 to 1×10^6 NK cells and RPMI-1640/10% FBS medium to a total volume of 2 ml into the tube (see Notes 39 and 40).
4. Add 100 IU/ml IL2.
5. Incubate at 37°C in 5% CO_2 for 24 h.
6. Centrifuge and remove the supernatant (see Note 41).
7. Resuspend the pellet with RPMI-1640/10% FBS medium.
8. Add the vector supernatant, resuspended NK cells and RPMI-1640/10% FBS medium to total 2 ml into a new fibronectin fragment-coated tube.
9. Add 100 IU/ml IL2.
10. Incubate at 37°C in 5% CO_2 for 24 h.
11. Centrifuge and resuspend the cells with 2 ml of RPMI-1640/10% FBS medium.
12. Transfer the cells to a well of a 24-well plate and add 100 IU/ml IL2.
13. Incubate at 37°C in 5% CO_2 for 2 days (see Note 42).
14. Replace half of the medium with 200 IU/ml IL2 and incubate at 37°C for 2 more days.
15. Assay for the transgene expression (see Note 43).

4. Notes

1. Our experience with anti-CD19-BBζ mRNA electroporation in NK cells is limited to the MaxCyte instrument, which is cGMP compliant. Other electroporation systems are commercially available.
2. NK cells expansion is generally superior with SCGM than with RPMI-1640 (20).
3. The K562-mb15-41BBL cells must be irradiated before coculture with PBMC. If an irradiator is not available, the K562-mb15-41BBL cells can be treated with mitomycin C (80 μg/ml for 1 h (47)) or another antimitotic to prevent cell division.

4. NK cells must be cultured at cell densities that allow cell–cell contact. We usually transfer 2 ml of the mixed diluted cells to a well in a 24-well plate at a final concentration of 1.5×10^6/ml for PBMC and 1.0×10^6/ml of K562-mb15-41BBL cells per well.
5. When PBMC are cultured with irradiated K562-mb15-41BBL cells in a flask or a bag, add the same volume of tissue culture medium plus 10% FBS and 20 IU/ml IL2 on days 2, 4, and 6.
6. After 7 days of coculture, there should be no viable K562-mb15-41BBL cell due to the combined effect of irradiation and cytotoxicity by expanded NK cells.
7. T cells do not expand in this culture system. After 7 days of coculture, the percentage of NK cells among PBMC increases to up 90%.
8. NK cells continue to expand in culture for 1 or 2 more weeks. The cells should be monitored daily for changes in the medium pH. The medium might need to be changed more often than described above. The cells must be split if the cultures appear to be too crowded.
9. After CD3+ T-cell depletion, the percentage of NK cells should be more than 90%. If a higher purity is required, then B cells and monocytes should also be eliminated using specific antibodies (NK cell isolation kit (Miltenyi; 130-090-864)) and magnetic beads. Alternatively, CD3+ T cell depletion can be followed by positive selection of CD56+ cells (CD56 Micro Beads, Miltenyi; 130-050-401). Various kits for selection are available from several manufacturers.
10. Confirm that the cleavage is complete by agarose gel electrophoresis; even a small amount of circular plasmid can affect the synthesis of the desired mRNA.
11. Various kits for synthesis of RNA with both a cap structure and poly (A) tails are available from several manufacturers, for example mScript mRNA Production System, Epicentre Biotechnologies, Madison, WI. We routinely use mMESSAGE mMACHINE T7 Ultra Kit, Ambion.
12. Use RNase-free pipette tips and perform the following steps in RNase-free area.
13. Avoid drying the RNA pellet completely, e.g., by air-drying for longer than 10 min, as it may become insoluble.
14. The yield of mRNA is expected to be about 30 μg.
15. In our experience, stimulation with 1,000 IU/ml IL2 is not mandatory if NK cells are electroporated within 3 days after expansion, as NK cells are still in an activation state.

16. Several manufacturers provide appropriate electroporation buffers.
17. Use RNase-free pipette tips in the following procedures.
18. DNA can be used instead of mRNA but electroporation efficiency with mRNA is higher than that with DNA (41).
19. The voltage and capacitance are critical parameters for high transfection efficiency. Electroporators typically have several program settings. Compare the settings and choose the optimal one. With the MaxCyte electoporator, we use the program "Static NK #2".
20. Protein expression is detectable approximately 4 h after electroporation. Expression is transient. With anti-CD19-BBζ mRNA, the corresponding receptor reaches maximum expression after 24 h, remains stable for up to 48 h after which expression declines progressively, becoming essentially undetectable after 5 days.
21. 293 T cells should be cultured in DMEM/10% FBS medium for at least 1 week after thawing. Because overconfluence leads to formation of cell clumps that can result in a less efficient transfection, 293 T cells must be split before they reach 80% confluence by detaching with 0.05% trypsin/EDTA.
22. Avoid contact of the cationic lipid transfection agent with the wall of the tube.
23. Because 293 T cells are easily detached, the medium must be removed or added very gently.
24. The retroviral vector supernatant can be filtered through a 0.45 μm filter to remove cellular debris, instead of being centrifuged.
25. The production of retroviral vector particles peaks 2 or 3 days after transfection and drops significantly 4 days after transfection.
26. Low viral titer is often caused by inefficient transfection of 293 T cells. Anticipated transfection efficiency of 293 T cells is over than 60%. If desired, collect the transfected 293 T cells and assay for the transgene expression, after collecting the last vector-containing supernatant.
27. Because the viral vector particles may be produced at this point, treat the removed medium with chlorine bleach at a 10% final concentration for 20 min before discarding. Tips, pipettes, and all other labware in potential contact with the retroviral vector supernatant must be also treated with chlorine bleach. At the end of each procedure, clean the work area with 70% ethanol.
28. Several methods have been developed to concentrate retroviral vector supernatants. PEG-it Virus Precipitation Solution

(System Biosciences), described in this protocol, does not require purification of the concentrated vector supernatant. By contrast, some methods for concentration require additional steps for purification prior to use; concentrated vector supernatant may contain contaminants that can be toxic to target cells.

29. A white pellet of viral vector particles should be visible at the bottom of the tubes after centrifugation.
30. This volume could vary depending on the number of cells to be transduced and the viral titer. The concentrated vector supernatant should be aliquoted and stored at −80°C. Avoid repeated freezing and thawing.
31. NIH/3T3 cells (ATCC #CRL-1658) are also frequently used instead of HeLa cells for determining viral titers.
32. Methods for assessment of transgene expression vary depending on viral vector. If the vector contains GFP or another marker which can be detected by flow cytometry, test transduction efficiency of target cells by flow cytometric analysis 4 days after transduction, when all transduced cells should be expressing GFP or their marker at detectable levels. Calculate the viral titer using the following formula.

$$\text{viral titer (IU / ml)} = \frac{\text{number of HeLa cells} \cdot \text{ percentage of transduced cells}}{\text{volume of vector supernatants (ml)}}$$

If the vector contains a drug resistance gene, split infected cells into selection conditions with the drug, count colonies 7–10 days after transduction and calculate the viral titer.

33. Non-coated tissue culture plates or dishes can be used instead of tubes. It is recommended to add 400 μl into each well of a 24-well plate or 2 ml into each well of a 6-well plate, respectively.
34. Alternatively, the tube can be allowed to stand for 2 h at room temperature.
35. It is recommended to add 400 μl into each well of a 24-well plate or 2 ml into each well of a 6-well plate, respectively, for blocking.
36. The fibronectin fragment-coated tubes can be stored up to 4 weeks.
37. NK cells can be transduced by centrifuging in the presence of polybrene and vector supernatant without the fibronectin fragment-coated tube or plate. However, the transduction efficiency is typically higher by incubating the cells and vector particles on the fibronectin fragment-coated vessels.
38. It is recommended to add 600 μl for each well of a 24-well plate or 3 ml for each well of a 6-well plate for rinsing.

39. Add the vector supernatant immediately after thawing.
40. Instead of adding both target cells and vector particles into fibronectin fragment-coated tube simultaneously, it is possible to add the vector supernatants to a fibronectin-coated tube first and incubate for 4 h at 37°C. Following aspiration of the supernatant, wash the tube with PBS and then add target cells with RPMI-1640/10% medium.
41. A second hit is not necessary, although it may increase transduction efficiency. If desired, procedures from step 8 to step 11 can be skipped.
42. The cells should be monitored daily during culture. The medium must be changed earlier than described above if it becomes acidic. The cells must be split if they appear too crowded.
43. Transgene expression after retroviral transduction is stable. However, because non-transduced cells tend to expand more than transduced cells, the percentage of transduced cells may decrease gradually. Use NK cells which are not cultured for a long time after transduction, or sort the transduced cells.

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Chapter 14

Reprogramming to Pluripotency and Differentiation of Cells with Synthetic mRNA

Peizhe Wang and Jie Na

Abstract

Changing cell fate without altering its genome is very desirable in many experimental systems and for cell therapy. Compared to DNA plasmid or viral-based approach, mRNA has the advantage of high transfection efficiency, no danger of changing the genome or creating mutational insertions. Here, we describe a straightforward protocol to synthesize mRNA of genes of interest, and use them to induce pluripotency and direct cell differentiation.

Key words: mRNA, Reprogramming, Differentiation, Embryonic stem cells, In vitro transcription

1. Introduction

An approach to efficiently direct cell fate change without genetic modification would be very useful for biomedical research and regenerative medicine. mRNA-mediated transient gene expression has been widely used in developmental biology studies. In 1971, Gurdon and colleagues demonstrated that in vitro synthesize mRNA can be translated into protein by *Xenopus* oocytes (1). Since then, microinjection of mRNA into *Drosophila*, *Xenopus*, zebra fish and mouse preimplantation embryos has become a major approach to study gene function and manipulate cell lineage in these model systems (2–4). Besides developmental biology research, mRNA transfection has also been used to modulate immune response (5–8). Several studies showed that antigen-presenting dendritic cells loaded with mRNAs of viral antigens were able to stimulate robust and specific T cell response toward the same virus (9, 10). More importantly, cells

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transfected with mRNA are safe for use in humans. A phase I/II clinical trial showed that autologous dendritic cells transfected with mRNA extracted from a patient's melanoma cells as vaccine produced specific response towards his/her own melanoma without any adverse effect (11).

Recently, several papers reported generation of transgene-free induced pluripotent stem cells (iPS cells) using synthetic mRNA (12–14). This is a significant step forward from previous DNA-based methods, in which foreign genetic materials either permanently left in the genome of iPS cells or later removed or lost after multiple rounds of cell division. In those cases, stringent genome-wide analysis needs to be carried out to confirm the absence insertional mutation in the genome (15, 16). The mRNA-based reprogramming approach has several advantages: first, it does not cause any genetic change in the host genome. Second, mRNAs are quickly translated into proteins with appropriate mammalian posttranslational modifications. This would result in much higher functionality than recombinant proteins produced in the bacteria. Third, single-stranded mRNA molecules are much smaller than double-stranded plasmid DNA molecules; therefore they can be introduced into cells with higher efficiency. Cell condition, transfection reagents, comparison of cell death after DNA and RNA transfection. Finally, it is easy to multiplex and control protein expression level by combining several different mRNAs of different concentrations. The disadvantage of mRNAs is that they are degraded after 2–3 days so that the expression window is relatively short. As generation of iPS cells often requires 2–3 weeks of stable expression, repeated mRNA transfection is required to induce pluripotency from somatic cells. This lead to another problem: unmodified mRNA can trigger strong interferon reaction in transfected cells and subsequent growth arrest (12, 14). Thus, for multiple rounds of transfection, it is necessary to use modified mRNA that can evade the cell's innate immune response machinery and recombinant protein B18R to sequester secreted interferon from cells. (12). Comparing to reprogramming, embryonic stem (ES) cell differentiation is a step-wise process; each step only takes a few days depending the desired cell type. Thus, mRNA transfection could be a very useful tool to direct cellular differentiation and generate functional cells that can be used in cell transplantation therapy.

Here, we describe a simple, straightforward protocol to synthesize mRNAs of gene of interest and transfect them into mammalian cultured cells to mediate reprogramming or differentiation. Depending on the cell type, the transfection parameters and differentiation conditions need to be optimized to fit individual experiment.

2. Materials

2.1. Cells

MRC5 (human fetal lung fibroblast cells) and HFF (human foreskin fibroblast cells) were purchased from ATCC. They were maintained in DMEM supplemented with 10% FCS (Gibco/Invitrogen) and 1× penicillin/streptomycin (Invitrogen).

HUES1 human ES cell line was obtained from Harvard University Stem Cell Center.

2.2. Reagents and Equipment for DNA Template Preparation

1. Restriction enzyme to linearize the plasmid containing the target gene.
2. DNA cleanup column.

   PCR product cleanup columns from QIAquick PCR Purification Kit (Qiagen) or GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) can be used.
3. 3M pH 5.3 Sodium acetate, store at room temperature.
4. 70% Ethanol made with molecular biology grade water, store at 4°C.
5. Molecular biology grade water (DNase and RNase free, store at 4°C) for all reactions and suspension solutions.
6. Thermostat (water bath or PCR machine), microcentrifuge.

2.3. Reagents and Equipments for mRNA In Vitro Transcription and Purification

1. Messenger RNA in vitro transcription kit.

   It is essential to use kits that produce RNA with a methylated cap analog (m7G[5′]ppp[5′]G) on the 5′ end. Capped mRNAs are translated much more efficiently, while uncapped mRNA gets degraded in cells rapidly.

   We often use AmpliCap-Max™ T3/T7/SP6 High Yield Message Maker kit (Epicenter), mScript mRNA production system (Epicenter), Ambion mMESSAGE mMACHINE kit.
2. RNA cleanup columns

   MicroSpinTM columns S-200 (Catalog number 27-5120-01, GE), store at 4°C.

   Or Mini Quick Spin Column (Catalog number 11814427001, Roche), store at 4°C.
3. Tris-saturated phenol–chloroform, pH 8.0. Store at 4°C and keep in dark place.
4. 3M pH 5.3 Sodium acetate, 70% ethanol and molecular biology grade water. Same as in Subheading 2.1.
5. PCR machine and refrigerated microcentrifuge.
6. Optional:

   3′-0-Me-m7G(5′)ppp(5′)G Anti-Reverse Cap Analog (ARCA) (NEB S1411L).

Pseudouridine (psi), 5-methylcytidine (5mC) from TriLink Biotechnologies, San Diego, CA.

Antarctic phosphatase (NEB). In our hands, mRNA with ARCA cap, psi, and 5mC did not give significant higher GFP or reprogramming factor protein expression, however, the mRNA yield was lower. Their benefit seems to be the lower cytotoxicity towards cells (see Note 4).

2.4. mRNA Transfection Reagents and Equipments

1. Tissue culture grade phosphate buffer saline (PBS) without Ca^{2+} and Mg^{2+}, store at room temperature.
2. Fibroblast Medium: DMEM (Invitrogen) with 10% fetal bovine serum, 1× GlutaMAX-I (Invitrogen) and 1× penicillin/strepcillin (Invitrogen), store at 4°C.
3. 0.25% Trypsin/0.2% EDTA, store at 4°C.
4. Electroporation system. We use Neon® Transfection System (MPK5000, Invitrogen) in our experiments. BTX ECM830 (BTX) with Cell Projects buffer or Nucleofector (Lonza) can also be used, but they caused more death in fibroblast cells.
5. TransIT-mRNA transfection Kit (Mirus Bio) or Lipofectamine 2000 (Invitrogen).

2.5. Small Molecules

1. Valproic acid (Merk).
2. BIX01294 (a G9a histone methyltransferase inhibitor) (Tocris).
3. 5-aza-2′-deoxycytidine (Sigma).

3. Methods

3.1. Prepare Template DNA for In Vitro Transcription of mRNA

1. Clone the coding region of the gene of interest and place it in between the 5′UTR and 3′UTR sequence of the RN3P plasmid (Fig. 1).
2. Linearize 10 μg RN3P plasmid containing the target gene with *Sfi*I (in our case) in a 50 μL reaction system. Incubate at 50°C for 2 h to ensure complete cut (see Note 1).
3. Purify *Sfi*I digested DNA with a PCR cleanup Kit.
4. Add 0.1 volumes of 3 M pH 5.3. Sodium acetate, mix well, then add 2.5 volumes of 100% ethanol and mix vigorously. Leave in −20°C for at least 2 h to precipitate DNA.
5. 4°C Centrifuge at 13,000 × *g* for 20 min to pellet the DNA, remove the supernatant, rinse DNA pellet with 70% ethanol once.
6. Dry DNA in air for approximately 5–10 min.
7. Resuspend the linearized DNA pellet in water. Adjust the final concentration to 0.5 μg/μL and store in −80°C.

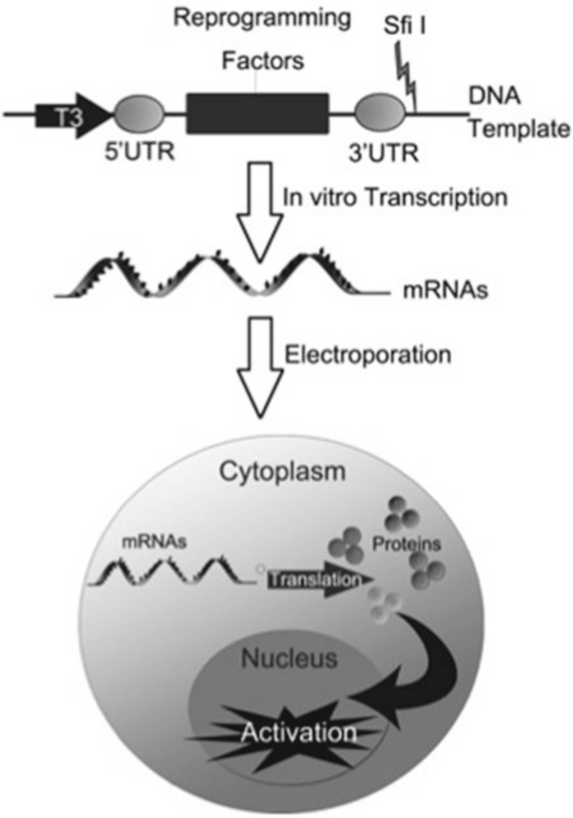


Fig. 1. Graphic presentation of using synthesized mRNA to activate gene expression in cells. The coding regions of reprogramming factors are cloned in between the 5′ and 3′ UTRs of the RN3P plasmid. In vitro transcription reaction is carried out to synthesize capped mRNA. After being microporated into the cell, mRNAs are quickly translated into proteins in the cytoplasm, then the reprogramming factor proteins enter the nucleus and induce cell pluripotency.

3.2. In Vitro Transcription of Capped mRNA (Fig. 1)

1. We often use AmpliCap-Max™ T3 High Yield Message Maker Kit (Epicenter) to make 5′ capped mRNA. Similar capped mRNA in vitro transcription kits, such as the mMESSAGE mMACHINE® Kit from Ambion, could also be used. The in vitro transcription reaction is set up following manufacturer's instruction in a 200 μL PCR tube (see Note 4).
2. Incubate the reaction at 37°C for 1 h in a PCR machine.
3. Add 1 μL of RNase-free DNase (come with the kit) to degrade the DNA template, mix gently and incubate in 37°C for 15 min. It has been reported that 5′ triphosphate of the in vitro synthesized mRNA can trigger cytotoxicity, thus we recommend adding antarctic phosphatase together with DNase to eliminate the 5′ phosphate (12). We have tried treating mRNA made with ARCA cap, psi and 5mC with antarctic phosphotase and found this caused noticeable less cell death. After DNase and phosphatase digestion, add 30μL 4°C nuclease-free water to the 20 μL reaction to make up for the 50 μL minimal volume needed for the spin column and proceed to next step immediately.

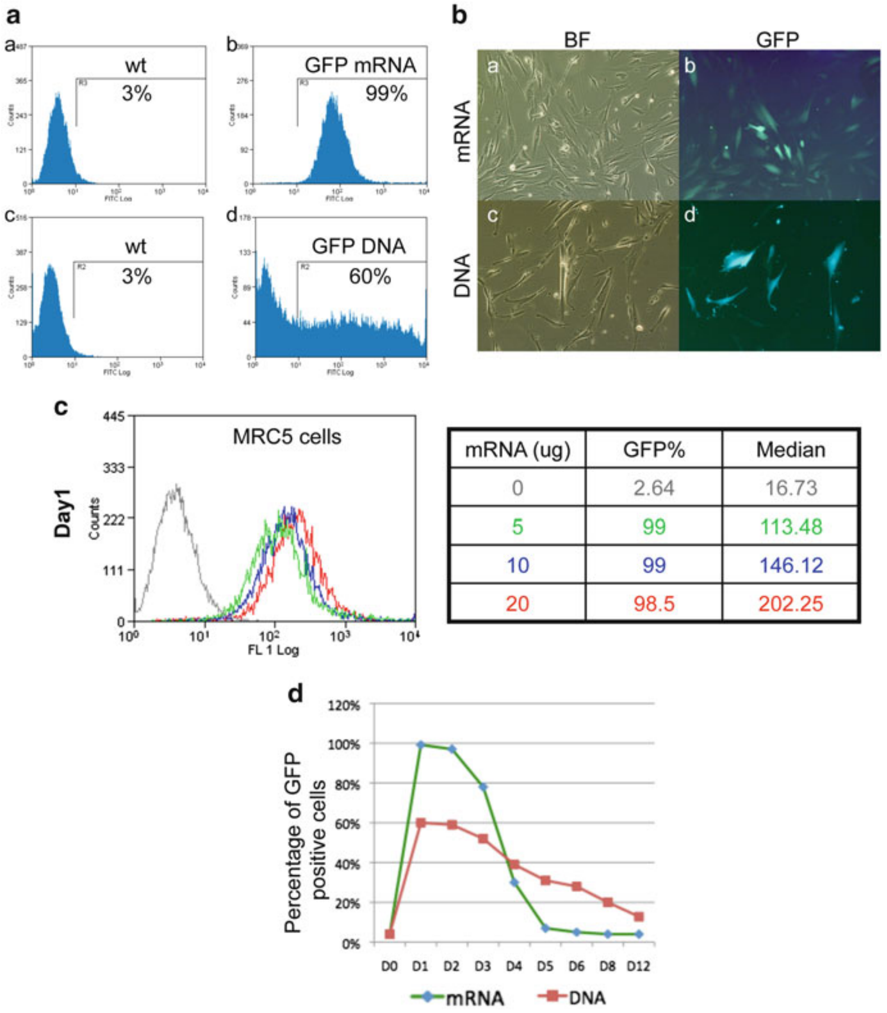
3.3. Purification and Storage of In Vitro Transcribed mRNA

The purpose of purification is to remove all the unincorporated nucleotide and excessive salt in the mRNA in vitro transcription reaction (see Note 2).

1. Take a mini Quick Spin Column (Roche) from 4°C, mix vigorously to resuspend the Sephadex matrix in the column buffer. Remove top cap, snap off bottom tip, and place the column into a sterile 1.5 mL tube. 4°C centrifuge at 1,000 × *g* for 1 min to pack the column and remove residual buffer.
2. Carefully apply 50 μL of mRNA reaction from Subheading 3.2 to the center of column bed. Centrifuge at 1,000 × *g*, 4°C for 4 min. Collect flow through into a sterile 1.5 mL tube, immediately put on ice.
3. Add equal volume of Tris-saturated phenol–chloroform (pH 8.0) and mix vigorously. Centrifuge at 10,000 × *g*, 4°C for 10 min.
4. Take the supernatant into a sterile 1.5 mL tube. Add equal volume of chloroform and mix vigorously. Centrifuge at 10,000 × *g*, 4°C for 10 min.
5. Take the supernatant into a sterile 1.5 mL tube, then add 0.1 volume of 3 M pH 5.3 Sodium acetate and 2.5 volumes of precooled 100% ethanol, mix vigorously and leave in −20°C for at least 2 h to precipitate mRNA.
6. Centrifuge at 13, 000 × *g*, 4°C for 20 min to pellet the mRNA, rinse with 70% ethanol once.
7. Dry mRNA in air for approximately 5 min (Don’t over-dry mRNAs as it will make them difficult to dissolve). Resuspend the pellet in water and determine the concentration. We typically obtain 40–60 μg of mRNA from a 20 μl reaction using the AmpliCap-Max™ T3 High Yield Message Maker Kit. Adjust the mRNA concentration to 2 μg/μL, aliquote and store in −80°C (see Note 2).

3.4. Electroporation of mRNAs into Fibroblast Cells for Reprogramming

1. Passage confluent fibroblasts at 1:2 ratio into T75 flasks the day before electroporation. To reprogram human fibroblast cells, we start from 5 to 10 million cells (see Note 3).
2. On the day of the experiment, renew the fibroblast medium 2 h before electroporation (see Note 3). Prewarm fibroblast medium in new T75 flasks in 37°C, 5% CO_2 incubator.
3. Briefly wash cells with PBS without Ca^{2+} and Mg^{2+} and detach them with 2 mL of 0.25% trypsin/0.2% EDTA.
4. Stop trypsin digestion with fibroblast culture medium and transfer cells into a 15 mL centrifuge tube. Centrifuge at 1,000 × *g* for 5 min to harvest cells.
5. Aspirate the medium and wash the cells once with 10 mL of PBS without Ca^{2+} and Mg^{2+}. Count the number of cells immediately.
6. Centrifuge cells down at 1,000 rpm for 5 min and remove PBS.



mRNA (ug)	GFP%	Median
0	2.64	16.73
5	99	113.48
10	99	146.12
20	98.5	202.25

Fig. 2. Efficient transfection of GFP mRNA into human fibroblast cells. MRC5 cells were used in this study. (**a**) FACS histogram of GFP positive cells after GFP mRNA or DNA transfection (b and d). Non-transfected cells (a and c). (**b**) GFP mRNA and DNA transfected cells. BF, brightfield; GFP, green channel. (**c**) Histogram and table of GFP positive cells after microporation of 0, 5, 10, and 20 μg of mRNA. (**d**) Percentage of GFP positive cells over 12 days following mRNA or DNA transfection. Originally published in Plews et al. PLoS ONE, 2010 (14).

7. Use Neon® Transfection System (Invitrogen) to microporate mRNA into cells according to manufacturer's instructions. Neon system has two microporation tip sizes, 10 and 100 μL. We resuspend cells at 10^7/mL in buffer R provided with the kit and use 100 μL tip for all our reprogramming experiments. This system is particularly effective for fibroblast cells. As seen in Fig. 2a, b, upon GFP mRNA transfection, 99% MRC fibroblast cells were positive for GFP, while only 60% of cells expressed GFP after DNA plasmid transfection. Moreover, the intensity of GFP fluorescence correlated with the amount of mRNA transfected (Fig. 2c). The GFP expression from mRNA was detectable until the 4th day after transfection (Fig. 2d). As the expression

level of each factor is important for the efficiency of reprogramming. After testing the effect of individual factor on fibroblast cells, we found that Oct4 promoted cell proliferation, while Sox2 inhibited it. Although SV40LT was reported to accelerate the pace of reprogramming, it can also inhibit p53 function and cause cell transformation. Thus we multiplexed mRNAs at the following amount: OCT4:SOX2:KLF4:cMYC:SV40LT (OSKMT) = 18:9:6:6:3 (μg/million cells) (14). Western blot analysis showed that in the first 2 days following microporation, fibroblast cells expressed more Oct4 and Sox2 proteins than hES cells did, while Myc proteins was at similar level as in hES cells. In HUES1 hES cells, Klf4 was below detection level. At day 3, the expression of Oct4, Sox2, and Klf4 dropped dramatically. cMyc maintained expression level until day 4, but decrease markedly at day 5 (Fig. 3a) (14). We found that the microporated mRNAs remained in cells for at least 3 days (Fig. 3b).

8. Transfer two millions of microporated cells into the prepared T75 culture flasks containing pre-warmed fibroblast medium.
9. Optional: as the protein of reprogramming factors only exists for no more than 3 days, after 3 days of culture, fibroblast cells may be transfected again as from step 2. This may produce more reprogrammed colonies but also results in significant cell death in some occasions.
10. After transfection, we switched to mouse fibroblast (MEF) conditioned human ES cell medium and added small molecules at following concentrations: valproic acid (200 μM), BIX01294 (1 μM), and 5-aza-2′-deoxycytidine (0.5 μM) as described in Plews (14) (see Note 5).
11. After 48 h, small molecules were removed and fibroblast was cultured in human ES cell medium until confluence (about 2 weeks). Then they were passaged onto gelatin-coated flasks and fed with MEF conditioned human ES medium to let reprogramming process to continue. Some small cell aggregates became visible 3 weeks after microporation (see Note 4).

3.5. Chemical Transfection of mRNA into Cells

Liposome-based chemical transfection reagents can also be used to deliver mRNAs into cells. We obtained about 50% and 30% positive rate with Lipofectamine 2000 (Invitrogen) and TransIT (Mirus Bio), respectively. However, in our hands, Neon microporator gave the best efficiency (up to 99%) and highest protein expression level. Cells transfected by microporation also grow better than cells transfected with Lipofectamine 2000.

3.6. Activation of Pluripotency Genes in Fibroblast Cells with mRNA

Although generation of iPS cells with defined factors takes 2–3 weeks (17–19), in the case of somatic nuclear transfer and ES-somatic cell fusion, pluripotency genes can be activated within 1–2 days (20–23). This suggests that a short exposure to

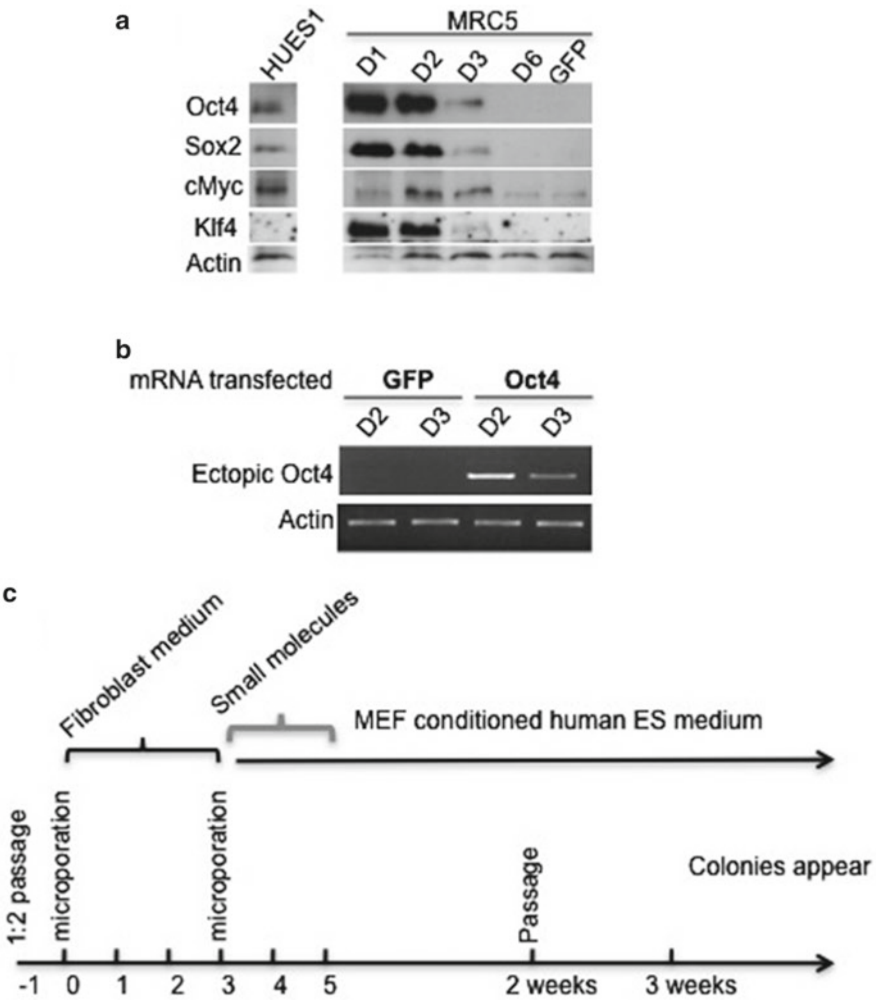


Fig. 3. (**a**) Protein expression following mRNA transfection. Western blot showing corresponding protein expression in 10^6 MRC5 cells transfected with OCT4 (17 μg), SOX2 (10 μg), cMYC (6 μg), KLF4 (6.5 μg), SV40LT (3.5 μg). The negative control is GFP mRNA transfected MRC5 cells. HUES1 is human ES cells. Originally published in Plews et al. PLoS ONE, 2010 (14). (**b**) RT-PCR result showing that transfected mRNA was still detectable 3 days after transfection, 20 μg of GFP and Oct4 mRNAs were microporated into 1 million MRC5 cells separately. Cells were lysed 2 and 3 days after microporation. Ectopic Oct4 mRNA could be detected in Oct4 transfected cells but not in GFP transfected cells. β-Actin was used as normalizing control. (**c**) Timeline of mRNA and small molecule reprogramming.

reprogramming factors combined with inductive environment may be sufficient to kick start the reprogramming process. Therefore, we designed the mRNA reprogramming procedure as described in Subheading 3.4 (Fig. 3b). We could detect the activation of endogenous pluripotency genes at day 3 (14). Small colonies appeared after 3 weeks. These colonies were positive for alkaline phosphatase (AP) activity and expressed Oct4 protein (14). Warren et al. reported efficiency as high as 4.4% using mixture of Oct4, Sox2, Klf4, cMyc, and LIN28 mRNAs in low-oxygen culture condition (12). Our efficiency is much lower, about 0.01%. This may be due

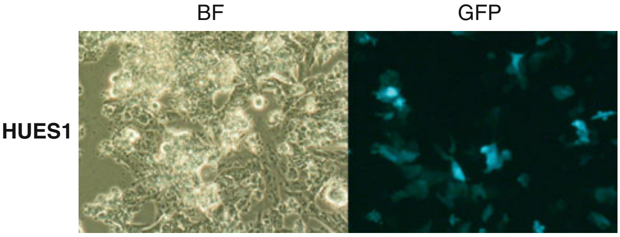


Fig. 4. GFP mRNA transfection in human ES cells. 10 μg GFP mRNA was electroporated into feeder-free cultured HUES1 human ES cells using BTX ECM830 and Cell Projects buffer. Images were taken 1 day after transfection. *BF* brightfield, *GFP* green channel.

to the short RNA expression window and normal oxygen culture condition in our experiments. The activation of innate immune response by large quantities of unmodified mRNAs could also impede the reprogramming process (12, 24) (see Note 4).

3.7. Direct Cell Differentiation with mRNA

Synthetic mRNA can also be used to promote embryonic stem cell differentiation. For human ES cells, we found that BTX ECM830 (BTX) and electroporation buffer (Cell Projects) gave better survival rate than Neon microporator. Using GFP mRNA as a marker, we observed over 80% of transfected HUES1 cells positive for GFP expression (Fig. 4a).

4. Notes

1. How to improve the yield of mRNA from in vitro transcription reaction.

   As reprogramming needs lots of mRNA, it is important to maximize the yield of your reaction. The quality of DNA template is crucial. To ensure the quality of the template, plasmid with the gene of interest must be linearized completely and with a 5′ overhanging end. RNase contamination may also lower the mRNA yield. We use PCR cleanup kit to purify the DNA templates and resuspend the linearized template with water and store them in −80°C. As mRNA is easily degraded in room temperature, it is good practice to put all reagents and mRNA reaction on ice whenever is necessary. The condition of in vitro transcription reaction is also important. We usually incubate the reaction for 1 h and then run 0.5 μL of the reaction on an agarose gel to check if good amount of mRNA has been made. If not, continue the reaction for another 30 min. If the reaction goes on for longer than 2 h, the newly synthesized mRNA may start to degrade. We also found that increasing the reaction temperature to 38°C can improve the yield.

2. Purification and storage of mRNA.
   mRNA for mammalian cell transfection needs to be of highest quality. In vitro transcription reaction contains large amount of free nucleotides and salt, which need to be cleaned out thoroughly. From our experience, the spin column is a quick and effective means to get clean mRNA. One should keep spin columns and all centrifugation steps at 4°C. The purified mRNA should be aliquoted and stored in −80°C, avoid repeated freeze and thaw.

3. Improve the efficiency of mRNA transfection.
   The viability of cells is crucial for transfection efficiency and reprogramming. We found that passaging fibroblasts the day before experiment and change medium 2–3 h prior to microporation can significantly increase the viability of cells and transfection efficiency. Before directing human ES cells differentiation with mRNA, cells should be cultured in feeder-free condition with minimal spontaneous differentiation, as both feeders and differentiated human ES cells may interfere with the differentiation process. Also, we found that transfection of human ES cells towards the end of their log phase growth gives better viability and efficiency.

4. Improve the stability and translation efficiency of mRNA.
   The adverse effect caused by repeated mRNA transfection is the biggest hurdle for reprogramming. Increase the stability of mRNA and its translation efficiency could permit less frequent transfection and reduce cellular stress. First, using the mRNA in vitro transcription system that produce capped mRNA is crucial for good protein translation. Second, substitution of the cytidine and uridine triphosphate with 5-methylcytidine and pseudouridine triphosphate during mRNA synthesis has also been show to increase the efficiency of mRNA translation and avoid the activation of innate immune response (12). In addition, the 5′ and 3′ UTR are both important for mRNA stability and translation efficiency. For example, we found that in human fibroblast cells, mRNA with only Kozak sequence ((gcc) gccRccAUG) in the 5′ UTR and a SV40 poly A tail at the 3′ end produced significant less protein than mRNA with a ribosome-binding element in the 5′ UTR and the *Xenopus* β-globin 3′UTR at the 3′ end.

5. The use of small molecules.
   Chemical inhibitors of epigenetic modifying enzymes have been shown to be able to significantly improve the efficiency for iPSC generation. In our reprogramming experiments with human fibroblast cells, we used HDAC inhibitor valproic acid (200 μM), G9a histone methyltransferase inhibitor BIX01294 (1 μM), and DNA methyltransferase inhibitor 5-aza-2′-

deoxycytidine (0.5 μM) (25, 26) and found that they significantly increased the level of endogenous pluripotency gene activation. However, the effect and the cytotoxicity of these small molecules may differ in different cell types. Thus, we recommend that one should first try a concentration gradient of these molecules on cells to be reprogrammed and find the most suitable concentration.

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Chapter 15

Antitumor Vaccination with Synthetic mRNA: Strategies for In Vitro and In Vivo Preclinical Studies

Mustafa Diken, Sebastian Kreiter, Abderraouf Selmi, Özlem Türeci, and Ugur Sahin

Abstract

Synthetic antigen-encoding mRNA is increasingly exploited as a tool for delivery of genetic information of complete antigens into professional antigen presenting dendritic cells for HLA haplotype-independent antigen-specific vaccination against cancer. Two strategies for mRNA-based antitumor vaccination have emerged into the clinical setting. One is transfection of autologous dendritic cells with synthetic mRNA for adoptive transfer into the patient. The other is direct injection of naked synthetic mRNA. Both methods have proven to be feasible and safe and to elicit antigen-specific immune responses. The design of novel synthetic vaccines employing synthetic mRNA requires further in-depth investigation of its bioavailability and immune pharmacology. This chapter summarizes the state-of-art in this field and describes methods elementary for preclinical studies of mRNA-based antitumor vaccine protocols.

Key words: mRNA vaccination, Dendritic cell, Cancer immunotherapy, Lymph node

1. Introduction

The pioneering work of Wolf et al. was the first to show in mice that intramuscularly injected mRNA coding for reporter genes such as luciferase or β-galactosidase results in detection of their gene products, thereby suggesting uptake and translation of the RNA by mouse muscle cells (1). Subsequent mouse studies demonstrated that immune responses can be raised against the RNA-encoded tumor antigen (2, 3). This process is very efficient as mRNA not only delivers the antigen but also activates dendritic cells (DCs) by providing costimulatory signals via Toll-like receptors TLR3, TLR7, and TLR8 (4). This intrinsic adjuvant activity

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combined with its transient and safe expression recommend antigen-encoding mRNA as a promising drug compound with a favorable safety and efficacy profile.

The first RNA vaccine studies utilized total mRNA isolated from tumor cells via basic molecular biology tools (5, 6). Such mRNA represents the whole repertoire of the tumor cell transcriptome and thus the entire spectrum of tumor-associated antigens. Disadvantages of this approach are that the composition of such a preparation is not well defined, that the amount of relevant individual mRNAs may be too low and that self-antigens within the mRNA pool may trigger autoimmunity. Meanwhile, synthetic mRNAs encoding defined antigens and obtained through in vitro transcription (IVT) have emerged for mRNA-based tumor immunotherapy. Highly pure synthetic mRNAs can be produced in large amounts which facilitates establishment of a production process in line with good manufacturing practice (GMP) and guaranties batch-to-batch reproducibility.

The main hurdle for in vivo application of mRNA as a vaccine format is its short extracellular half-life. The rapid degradation of mRNA molecules by ubiquitous extracellular ribonucleases constrains pharmacologically effective dosing of mRNA. The two strategies currently evaluating mRNA-based vaccines in human clinical trials address this challenge in different ways. One approach is to electroporate antigen-encoding synthetic mRNA into autologous DCs, thereby protecting it against degradation. The other resorts to pharmacologically stabilizing synthetic mRNA for direct injection.

The capability of DCs to take up RNA, they are coincubated with and to present the antigen for in vitro and in vivo induction of immune responses has been first shown by Boczkowski et al. (7). The follow-up studies employed electroporation as an efficient method to deliver RNA into DCs (8, 9). Since then various approaches have been explored to improve mRNA-engineered DC vaccines. Pretreatment of the DC administration site with inflammatory cytokines provided in vivo stimuli for DC maturation and migration to lymph nodes. Alternatively, mRNA transfected DCs were injected into lymph nodes ensuring their availability at the site of T cell priming (10). Vaccination of mice with DCs transfected with a combination of mRNAs coding for tumor antigens or antigens involved in neovascularization showed synergistic effects and slowed down angiogenesis and tumor growth (11). Intravenous or intradermal transfer of autologous monocyte-derived DCs transfected with mRNA coding for PSA into prostate cancer patients evoked T cell immunity without severe adverse effects providing the proof-of-principle for human use (12). Several other clinical trials using different antigens with or without adjuvant combinations provided encouraging data for the potency of this approach in treating cancer (13).

Direct injection of naked mRNA is an attractive alternative to adoptive cell transfer as it bypasses the laborious generation and transfection of DCs in vitro.

Even though vaccination with naked mRNA was one of the earliest application strategies, it took several improvement cycles addressing the challenges of mRNA pharmacokinetics. Several strategies based on modification of the mRNA sequence have been proposed to increase its half-life and optimize its translational characteristics. The addition of 3′ untranslated regions (UTRs) of α and β globin genes of human or *Xenopus laevis* origin to the plasmid DNA template has been reported to increase the stability of synthetic mRNAs (14). Mice receiving intramuscular injection of naked globin UTR-stabilized synthetic RNA coding for carcinoembryonic antigen (CEA) was able to generate anti-CEA antibodies (2). In an another study, direct injection of naked globin UTR-stabilized β-galactosidase synthetic mRNA into the ear pinna of mice resulted in the induction of antigen-specific antibody and $CD8^+$ T cell responses (3). Combination of this approach with elongation of the poly(A)-tail from 70 to 120 bp further increased stability and translation efficiency of mRNA (15). Only half of the RNA molecules capped with the standard cap analog m7G(5′)pppG carry it in the correct direction and can be translated. The use of modified cap analogs such as the anti-reverse cap analog (ARCA) or its variant beta-S-ARCA-D1, which are always incorporated in the correct direction, resulted in higher translation efficiency of mRNA (16, 17). Beta-S-ARCA-D1 has been shown to result in enhanced expression of the antigen for longer periods of time as well as a higher magnitude of immune responses as compared to ARCA (18).

Modification of treatment protocols further improved the outcome. Combination of GM-CSF treatment with naked mRNA vaccination was shown to enhance the humoral response and polarize it to cellular immune responses (14). Moreover, direct intranodal injection of immune-pharmacologically optimized antigen-coding mRNA was shown to be more efficient than other application routes in eliciting protective and therapeutic antitumoral immunity (19). Combining mRNA administration into the lymph node with Fms-like tyrosine kinase 3 (FLT3) ligand as an adjuvant further enhanced immune responses (20). The feasibility and safety of direct vaccination with naked synthetic mRNA for human use was demonstrated in clinical trials which achieved induction of antigen-specific humoral responses and T cell immunity in some patients (21–23). The emerging understanding of the mechanism underlying the uptake of mRNA will contribute to paving the way for better design of vaccines based on naked antigen-encoding mRNA (24).

The full potential of mRNA as vaccine format is still not fully exploited. In this chapter, we provide protocols for key methods required for preclinical studies of mRNA-based vaccine strategies: (1) transfection of synthetic mRNA into mouse bone-marrow-derived DCs (BMDCs) via electroporation for in vitro or in vivo use, (2) intranodal immunization of mice with naked antigen-coding synthetic mRNA, (3) passive pulsing of DCs with synthetic mRNA, (4) tetramer technology to monitor elicited immune responses in blood samples.

2. Materials

2.1. mRNA Transfer into Bone-Marrow-Derived DCs

1. Electroporator Gene Pulser II (BioRad).
2. Electroporation cuvettes (0.4 cm electrode) (BioRad).
3. PBS + 2 mM EDTA.
4. X-Vivo 15 serum-free medium (Biowhittaker).
5. Complete DC medium: RPMI 1640 medium with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, and 10% heat inactivated FCS supplemented with 10% (v/v) supernatant of a GM-CSF producing cell line (X6310) (corresponds to 200 U/ml rmGM-CSF).
6. RNase-free pipette tips.
7. RNaseZap (Ambion).
8. 1 μg/ml Propidium iodide (Sigma) or 0.1 μg/ml 7-AAD (Invitrogen) solution in PBS.
9. In vitro transcribed mRNA coding for enhanced green fluorescent protein (eGFP-RNA) (15).
10. 4- or 6-color flow cytometry (FACS) system.

2.2. Intranodal Immunization with Naked mRNA

1. Ketamine (Ratiopharm).
2. Xylazine (Bayer Healthcare).
3. 70% Ethanol.
4. Surgical equipment (scissors, forceps) for small laboratory animals.
5. 10× RNase-free PBS.
6. 0.3-ml Syringe with an ultrafine needle (31G).
7. Michel wound clips (Perfect Agrafes Chirurgicales).

2.3. Passive Pulsing of DCs with mRNA

1. PBS+2 mM EDTA.
2. X-Vivo 15 serum-free medium (Biowhittaker).
3. 10× RNase-free PBS.

4. RNase-free pipette tips.
5. Thermal block.
6. RNase-free Eppendorf tube.
7. In vitro transcribed mRNA coding for Influenza Hemaglutinin and labeled with Cy3 nucleotides (Cy3-RNA) (24).
8. In vitro transcribed mRNA coding for firefly luciferase (Luc-RNA) (18).
9. Luminescence reader (Tecan).

2.4. Monitoring of Elicited Immune Responses

1. EDTA or Heparin containing tube.
2. FACS tubes.
3. APC labeled MHC class I tetramer (iTAg *Tetramer*/APC—H-2 Kb OVA-SIINFEKL (Cat. Number T03002) (Beckman Coulter)).
4. Lymphocyte-specific monoclonal antibody (FITC conjugated anti-mouse CD8a monoclonal antibody (clone 5H10)) (Cat. Number MCD0801) (Invitrogen).
5. FACS lysis solution (10×) (Catalog No. 349202) (BD Biosciences).
6. ELB (Erythrocyte Lysis Buffer): 1× FACS Lysis Solution made with dH_2O.
7. RNase-free Eppendorf tube.
8. In vitro transcribed mRNA coding for SIINFEKL epitope of chichem ovalbumin (SIINFEKL-RNA) (19).
9. 4- or 6-color flow cytometry (FACS) system.

3. Methods

3.1. mRNA Transfer into Murine Bone-Marrow-Derived DCs

1. Clean all pipettes, pipette tips as well as working space with RNaseZAP to eliminate RNases.
2. Place electroporation cuvettes on ice.
3. Harvest bone-marrow-derived DCs (BMDCs) (see Note 1) and centrifuge at 300 × *g* for 8 min (4°C).
4. Discard the supernatant and add 40 ml cold PBS-2 mM EDTA. Resuspend the cells by pipetting up and down (see Note 2).
5. Centrifuge at 300 × *g* for 8 min (4°C).
6. Discard the supernatant, add 40 ml cold X-Vivo 15 serum-free medium and resuspend the cells by pipetting up and down.
7. Count cells in a Neubauer chamber.

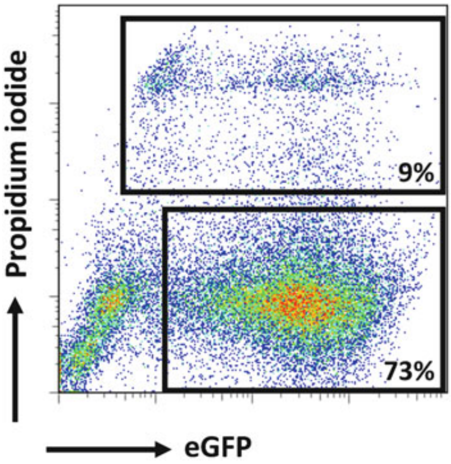

Fig. 1. Electroporation of BMDCs with synthetic eGFP-encoding mRNA. BMDCs were electroporated with 10 mg synthetic eGFP mRNA. Transfection efficiency (9%) as well as mortality (73%) was analyzed via FACS 24 h after electroporation.

8. Centrifuge at 300 × *g* for 8 min (4°C).
9. Discard the supernatant and resuspend cells ($1\text{–}2 \times 10^6/250\ \mu l$) in cold X-Vivo 15 (see Note 3).
10. Transfer 250 μl cell suspension to electroporation cuvettes.
11. Add synthetic mRNA encoding the antigen of interest (10–20 μg) and mix around 10 s by pipetting up and down with a 100 μl pipette (see Note 4).
12. Electroporate the cells by using Gene Pulser II at 276 V and 150 μF (see Note 5).
13. Transfer the cells to 6-well plates with complete DC medium (see Subheading 2.1) and incubate at 37°C and 5% CO_2.
14. Analyze transfection efficiency and mortality via FACS after 20–24 h (Fig. 1) (see Note 6).

3.2. Intranodal Immunization with Naked mRNA

See also Fig. 2 for illustration of the experimental procedure.

1. Anesthetize mice with a mixture of Ketamine (120 mg/kg body weight) and Xylazine (2 mg/kg body weight).
2. Sterilize the upper thigh area with 70% ethanol.
3. To surgically expose the target lymph node, identify the area where the upper thigh joins the lower abdomen. Use scissors to make a small incision into the skin to expose subcutaneous tissue.
4. Formulate the synthetic mRNA (10–30 μg) in an RNase-free Eppendorf tube (1.5 ml) by first mixing 10× RNase-free PBS and water in the tube and then adding mRNA to have a final 1× PBS solution with a final volume of 5–10 μl (see Note 7). Store mRNA solution on ice.

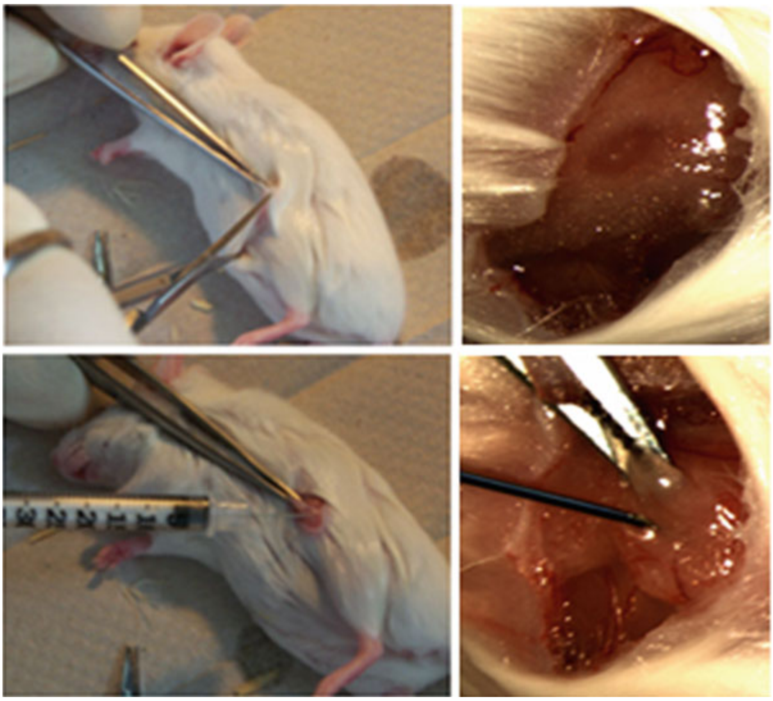

Fig. 2. Intranodal injection of naked antigen-encoding mRNA. An incision is made in the inguinal area (*upper left*) to surgically expose the lymph node (darker ellipse-like spot) (*upper right*). The mRNA solution is injected to the lymph node (*lower left*) as confirmed by enlargement of the lymph node (*lower right*).

5. Using (see Note 7) a single use 0.3-ml syringe with an ultrafine needle for intranodal administration, suck the mRNA solution into the syringe.
6. Locate the inguinal lymph node and use forceps to gently fix it in place.
7. Inject the mRNA solution to the inguinal lymph node (see Note 8).
8. Close the incision with Michel wound clips (see Note 9).

3.3. Passive Pulsing of DCs with mRNA

It is recommended to use immature DCs, which have the highest capability of mRNA uptake upon pulsing (Fig. 3).

1. Place electroporation cuvettes on ice.
2. Harvest DCs and centrifuge at 300 × *g* for 8 min.
3. Discard the supernatant and add 40 ml cold PBS-2 mM EDTA.
4. Centrifuge at 300 × *g* for 8 min (4°C).
5. Discard the supernatant and add 40 ml cold X-Vivo 15 serum-free medium to the cells.
6. Count cells in a Neubauer chamber.

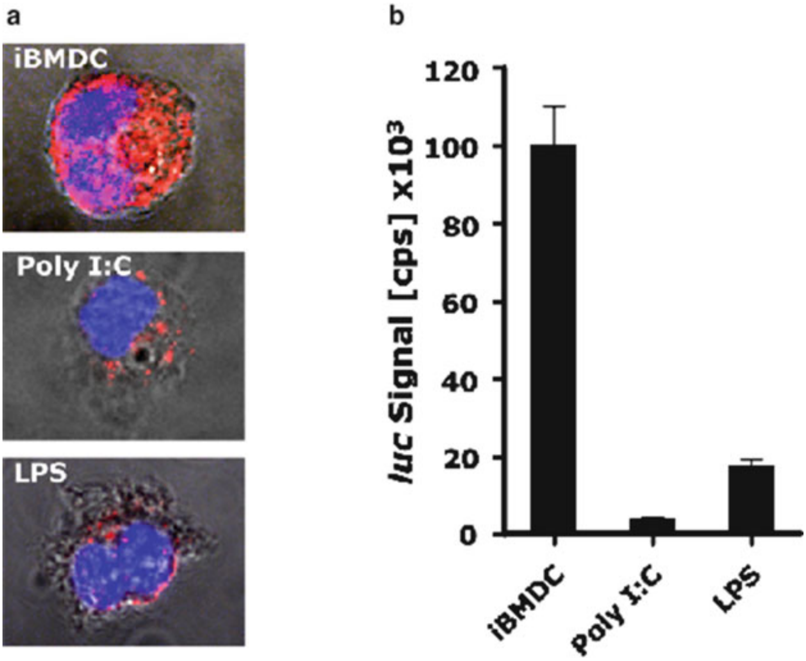

Fig. 3. Detection of pulsed synthetic mRNA in BMDCs. Immature BMDCs (iBMDC) and their mature counterparts generated by different maturation stimuli (Poly I:C, LPS) are analyzed by fluorescence microscopy (**a**) or luciferase assay (cps: counts per second) (**b**) following in vitro pulsing with Cy3-RNA (10 μg) and Luc-RNA (5 μg). (Cy3-RNA (*red*) and Hoechst 33342 counter staining of nuclei (*blue*)).

7. Centrifuge at 300 × *g* for 8 min (4°C).
8. Resuspend the cells in cold X-Vivo 15 to a cell density of $1–2 \times 10^6$/ml.
9. Transfer 1 ml of cell suspension to a 1.5 ml RNase-free Eppendorf tube (see Note 10).
10. To remove the culture medium completely, spin at 300 × *g* for 6 min at room temperature and carefully take the supernatant off with a Pasteur pipette. Quick spin DCs samples once more and remove any remaining trace amounts of fluid.
11. Place cell pellet samples into a preheated thermal block and wait for 2–3 min for equilibration of all samples to 37°C.
12. Dissolve the mRNA coding Firefly luciferase, and Influenza hemaglutinin RNA labeled with Cy3 fluorescent nucleotides (Cy3-UTP) (Cy3-RNA) in PBS (see Notes 7 and 11).
13. Add 10–20 μl of this mRNA formulation to each of the cell pellets and pipet up and down gently with a 100 μl pipette (see Note 12).
14. Incubate samples pulsed with mRNA in thermal block (37°C) for 15 min.

15. Add 500 µl FCS containing the required cell culture medium and incubate samples for 5 min.
16. Transfer cells to appropriate plates for further cultivation and analysis via luciferase assay (for synthetic mRNA coding for firefly luciferase) and fluorescence microscopy (influenza hemaglutinin mRNA labeled with Cy3 fluorescent nucleotides (Cy3-UTP)) (example provided in Fig. 3).

3.4. Monitoring of Elicited Immune Responses

1. Collect 100–150 µl blood by venipuncture into an EDTA or Heparin containing tube (see Note 13).
2. Transfer 100 µl blood to a clean FACS tube.
3. Add the SIINFEKL-specific MHC tetramer and the anti-mouse CD8 monoclonal antibody (see Note 14).
4. Vortex gently (2–3 s) and incubate 30 min in the dark at 4°C (see Note 15).
5. Add 300 µl ELB and vortex gently (2–3 s)
6. Incubate in the dark at room temperature for 5 min.
7. Add 3 ml PBS and centrifuge at 450 × *g* for 6 min.
8. Discard the supernatant.
9. Repeat steps 7 and 8.
10. Resuspend the cells in 200–300 µl PBS and store at 4°C until data acquisition via FACS (example provided in Fig. 4).

4. Notes

1. Murine BMDCs are generated according to published protocols (25). Bone marrow cells were obtained from femurs and tibias of mice and cultured using complete DC medium for 7 days with a cell density of 4×10^5 cells/ml in petri dishes (10 ml) to obtain BMDCs. The cultures were fed on day 2 and 5 with complete DC medium.
2. Resuspension of cells as required after each wash step should be performed diligently as avoidance of clumps is of importance. It is recommended to do this by carefully pipetting up and down.
3. Cell numbers can vary between 1×10^6 and 2×10^7 (Electroporation volume: 250 µl).
4. Avoid generation of air bubbles as they reduce transfection efficacy. It is important that steps 1–11 are processed swiftly without any interruptions. Otherwise BMDCs will have a reduced vitality compromising the efficacy of the whole procedure.

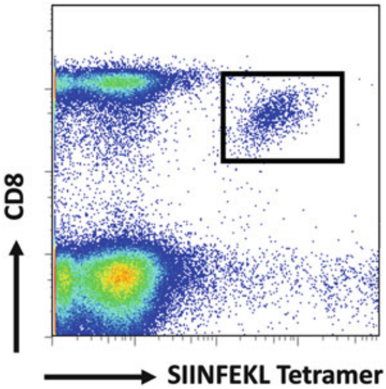

Fig. 4. Monitoring of immune responses elicited by intranodal immunization with naked antigen encoding synthetic mRNA using tetramer technology. C57BL/6 mice ($n = 3$) received two intranodal injections (d0, d3) of naked synthetic mRNA coding for the immunodominant epitope of chicken ovalbumin (SIINFEKL). SIINFEKL-specific CD8$^+$ T cells in peripheral blood are quantified by tetramer staining on day 8.

5. Electroporation parameters can vary based on the instrument used. It is recommended to determine optimal electroporation parameters in advance, which ensure high transfection efficiencies (70–80%) and low mortality rate (10–15%).
6. Synthetic mRNA coding for enhanced green fluorescent protein (eGFP) can be used to assess transfection efficiency. Mortality rate is determined via addition of propidium iodide or 7-AAD.
7. The amounts of 10× PBS and water to be added depends on the injection volume and the concentration/amount of synthetic mRNA therefore have to be calculated for each experiment. Other physiological buffers, such as 0.15 M NaCl, can also be used instead of PBS.
8. Enlargement of the lymph node upon injection can be regarded as a sign of proper injection.
9. Depending on the size of the incision, up to three Michel wound clips can be used.
10. At this stage, cells can be incubated with any compound that was supposed to influence the uptake of synthetic mRNA, for example, with an inhibitor of macropinocytosis.
11. Synthetic mRNA coding for eGFP or Influenza hemaglutinin RNA labeled with Cy5 fluorescent nucleotides (Cy5-UTP) can be pulsed into cells and analyzed via FACS.
12. For comparing different samples it is important to standardize the coincubation of cells and RNA. For example, a defined number of mixings should be applied in a defined time before placing the reaction tube into the thermal block.
13. For tetramer staining of cell preparations (e.g., cells from lymph nodes, spleen), prepare single cell solutions from tissue

following standard protocols, omit steps 1, 5, 6 and stain 1×10^6 cells/100 μl PBS.

14. MHC Tetramers and antibodies may be different from those specified here depending on the epitope and phenotype of T cells.
15. The incubation time and temperature can vary for different MHC tetramers. Some MHC tetramers might require special clones of anti-lymphocyte monoclonal antibodies and sequential staining with the MHC tetramer and the anti-lymphocyte monoclonal antibodies. Instructions of the supplier should be followed.

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Chapter 16

Mannosylated and Histidylated LPR Technology for Vaccination with Tumor Antigen mRNA

Chantal Pichon and Patrick Midoux

Abstract

mRNA-based vaccines are currently being developed for treating various diseases including cancers. For this purpose, synthetic or in vitro transcribed (IVT) mRNA encoding tumor antigen offers several advantages over plasmid DNA encoding tumor antigen including better delivery and security. In this chapter, we report the preparation of mannosylated mRNA nanoparticles termed mannosylated lipopolyplexes or Man-LPR loaded with mRNA encoding a melanoma antigen. This formulation enhances the transfection of dendritic cells (DCs) in vivo and the anti-B16F10 melanoma vaccination in mice. The mRNA is formulated with histidylated liposomes and a histidylated polymer. Those pH-sensitive vectors promote membrane destabilization in endosomes upon the protonation of their histidine groups, allowing nucleic acid delivery in the cytosol. To favor DCs targeting via the mannose receptor, a mannose lipid is incorporated in the liposomes. Here, we provide protocols for the preparation of mannosylated liposomes, the synthesis of mRNA, mice immunization based on systemic injection, measurement of the cellular immune response and determination of the number of transfected splenic DC.

Key words: mRNA, Transfection, Dendritic cells, Vaccine, Histidine, Liposomes, Polymer

1. Introduction

Dendritic cells (DCs) are the most important component of immunity, playing a critical role in antigenic presentation. Immunization of patients with genetic material encoding tumor antigen or with genetically modified DCs expressing tumor antigen are interesting vaccine strategies for cancer. The expression of exogenous genetic material encoding tumor antigen in DCs allows the antigen expression in its native form and presentation of all epitopes via the major histocompatibility complex (MHC) class I pathway, leading to the induction of tumor-specific cytotoxic T lymphocytes. In this

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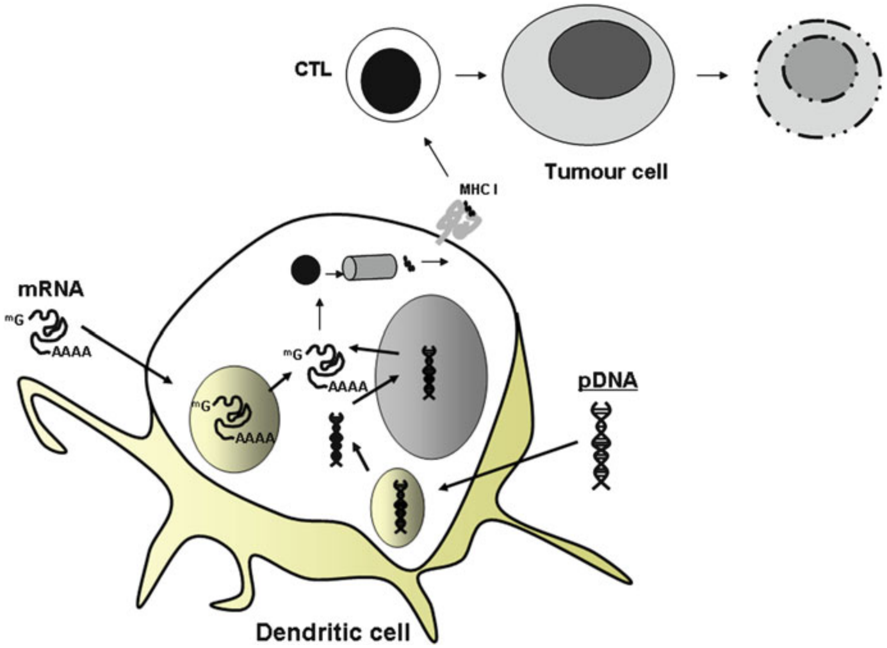


Fig. 1. mRNA-based transfection versus DNA-based transfection: The translation of mRNA occurring in the cytosol, the delivery of mRNA in the cytosol of dendritic cells is sufficient to produce the antigen By contrast, the production of the antigen from plasmid DNA requires the delivery of plasmid DNA in the nucleus which is surrounded by the nuclear envelope, a highly selective barrier. The expression of exogenous genetic material encoding tumor antigen in its native form allows the presentation by dentritic cells of all epitopes via the major histocompatibility complex (MHC) class I pathway and the induction of antigen specific cytotoxic T lymphocytes.

context, the transfection of DCs with in vitro transcribed (IVT) mRNA encoding tumor antigen offers several advantages over the corresponding plasmid DNA encoding tumor antigen. First, delivery of mRNA to translation machinery which occurs in the cytosol avoids the necessity to pass the selective barrier of the nuclear envelope. Furthermore, mRNA is quickly translated and allows a transient expression of the antigenic protein over a limited time (which can be, for example, only few hours). Therefore, the delivery of IVT mRNA into the cytosol of non-dividing cells is easier and sufficient than the delivery of plasmid DNA in the nucleus (Fig. 1). Most importantly, there is no uncontrolled long-term expression of the antigen as might occur upon gene integration in the genome, which could induce deregulation in gene expression and eventually lead to tumors. Moreover, IVT mRNA does not bear any regulatory nucleic sequences (promoters, terminators, enhancer, and so one) and antibiotic-resistance nucleic sequences that might be injected in the vaccinated population.

mRNA vaccines have been developed these last years (1–7). CureVac GmbH, a biotech company specializing in the development of mRNA vaccines has engaged a first phase I/IIa clinical trial to treat patients with prostate cancer and metastatic non-small-cell lung carcinoma. These mRNA vaccines appeared to trigger a

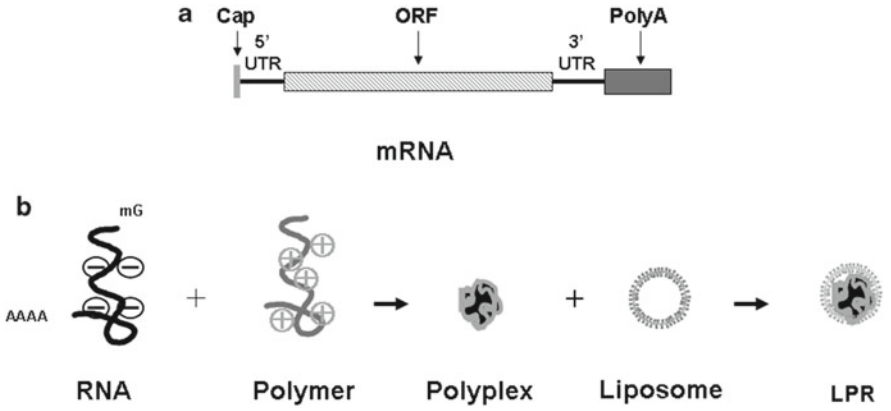


Fig. 2. (**a**) mRNA. In eukaryotic cells, mature mRNA has a tripartite structure consisting of a 5′ untranslated region (5′UTR), an open-reading frame (ORF) made up of triplet codons that each encodes an amino acid and a 3′ untranslated region (3′UTR). Cytoplasmic mRNA contains a cap structure at its 5′ terminus and a Poly(A) tail of 100–250 adenosine residues at its 3′ terminus. (**b**) LPR. Liposomes/Polymer/mRNA ternary complexes or lipopolyplexes: mRNA is condensed with a cationic polymer to form polyplexes, and liposomes are added to form mRNA lipopolyplexes or LPR.

specific and relevant immune response. The technology could also be used to develop prophylactic vaccinations against infections, where the production of protective antibodies plays a key role.

A high level of antigen protein upon mRNA transfection of DCs depends on the use of an optimal mRNA transcript. In eukaryotic cells, mature mRNA has a tripartite structure consisting of a 5′ untranslated region (5′UTR), an open-reading frame (ORF) made up of triplet codons series that code for each amino acid and a 3′ untranslated region (3′UTR) (Fig. 2a). This mRNA also contains a cap structure at its 5′ terminus and a Poly(A) tail of 100–250 adenosine residues at its 3′ terminus (8, 9). Recognition of the mRNA 5′ end and 40S ribosomal subunit recruitment requires the initiation factor eIF4F complex (10, 11). This complex assembles the cap-binding protein (eIF4E) and an RNA helicase (eIF4A) bound via eIF4G. The latter interacts also with the eIF3 complex, which binds the 40S ribosomal subunit directly. The mRNA 5′ and 3′ elements are recognized by initiation factors to allow ribosome binding and scanning along the 5′UTR. The length of the Poly(A) tail is important for both mRNA translation and mRNA stability. Poly(A) tail binds to the poly adenosyl binding protein (PABP) which interacts with the N-terminal part of eIF4G leading to mRNA circularization via the cap-eF4E-eF4G-PABP-Poly(A) complex (12). The Poly(A) tail can bind several PABP molecules and the binding of multimerized PABP to eIF4G can increase the affinity of eIF4E for the 5′ cap. This synergy between the Poly(A) tail and the 5′ cap is supposed to increase the expression at the level of translation.

A high level of antigen protein upon mRNA transfection of DCs depends also on the use of an efficient transfection reagent. mRNA complexed either with cationic liposomes, cationic peptides, or both of them have been used to immunize mice.

The vaccination capacity of mRNA has been compared in mice upon its administration as either as naked form, or as complexed with protamine sulfate, or as mRNA/protamine/liposomes ternary complexes (1). Both three mRNA forms allow induction of a humoral and cellular immune responses. Of note, the coadministration of naked mRNA and mRNA complexed with protamine sulfate allows a better immunization capacity and a better antitumor vaccination effect than the mRNA/protamine sulfate complexes alone, due to the adjuvant capacity of the mRNA via Toll-like receptors (13). In another application, the mRNA of Bax, a protein involved in apoptosis, complexed with DOTAP/DOPE liposomes has been shown to delay the tumor growth in nude mice transplanted with human malignant melanoma cells (14).

Liposomes/Polymer/mRNA ternary complexes termed lipoplyplexes (LPR) can be used to deliver mRNA encoding antigen in vivo (Fig. 2b). We have found that MART-1 mRNA LPR are an efficient system for anti-B16F10 melanoma mRNA-based vaccine in mice (15). The mRNA encoding MART-1 melanoma antigen is formulated with histidylated liposomes and a histidylated polymer. Those pH-sensitive vectors promote membrane destabilization in endosomes upon the protonation of their histidine groups, allowing nucleic acid delivery in the cytosol (16–18). First, mRNA is condensed with PEGylated histidylated polylysine (PEG-HpK) (Fig. 3) before adding liposomes made of l-histidine–(*N,N* di-*n*-hexadecylamine)ethylamide and cholesterol. The absence of any vaccine effect obtained when the mRNA was complexed with liposomes alone or with the cationic polymer alone demonstrated the efficiency of LPR formulation over those of lipoplexes or polyplexes.

To favor DCs targeting, we have prepared Mannosylated LPR (19, 20). Mannosylated liposomes are obtained by insertion of 11 mol% of a mannopyranosyl-glycolipid into the liposome formulation made with a cationic O,O-dioleyl-N-[3 N-(N-methylimidazolium iodide) propylene] phosphoramidate, lipid **1**, and a neutral *O,O*-dioleyl-*N*-histamine phosphoramidate, co-lipid **2**, in an equal molar ratio (21–23) (Fig. 3). Lipid **1** possesses an *N*-methylimidazolium polar head conferring a permanent positive charge. The imidazole group of co-lipid **2** can acquire a cationic charge when the pH of the medium drops below pH 6 favoring the endosome destabilization and nucleic acids delivery in the cytosol. We have demonstrated that in mice that have been intravenously injected with enhanced green fluorescent protein (EGFP) mRNA-loaded Man-LPR, their spleen had more DCs expressing EGFP than spleen of mice that have been injected with LPR (19). We have found also that mice immunization with MART-1 mRNA-loaded Man-LPR exhibited a greater inhibition of B16F10 melanoma growth than with MART-1 LPR1 where MART-1 (Melan-A) is the human melanoma antigen (19). These results indicate that tumor antigen mRNA-loaded Man-LPR is an efficient system to induce an anticancer immune response.

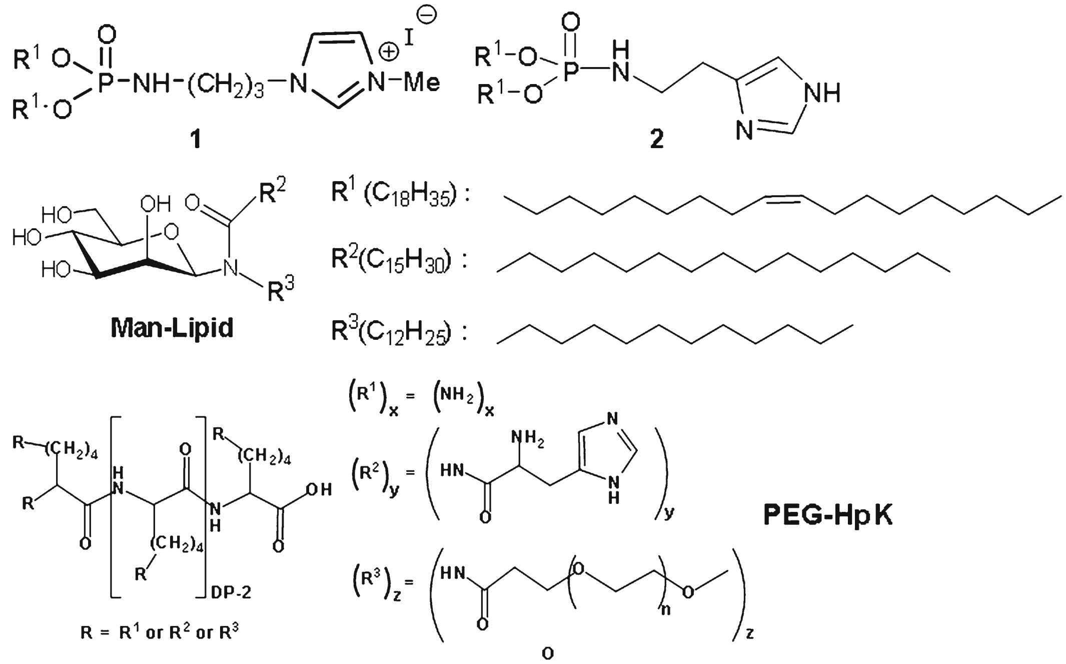


Fig. 3. Lipid and polymer structures. **1**: *O,O*-dioleyl-*N*-[3*N*-(*N*-methylimidazolium iodide)propylene] phosphoramidate; **2**: *O,O*-dioleyl-*N*-histamine phosphoramidate; Man-lipid: β-d-mannopyranosyl-*N*-dodecylhexadecanamide; PEG-HpK: PEGylated and histidylated polylysine.

In this chapter we present a general protocol that can be used as starting point for developing mRNA tumor vaccines. We provide protocols for polymer and lipid synthesis, liposomes preparation, mRNA production, mice immunization, and measurement of the cellular immune response.

2. Materials

2.1. PEGylated Histidylated Polylysine Synthesis

1. Poly-l-lysine, HBr (average molecular weight of 30,000–70,000; average degree of polymerization = 240; Cat # P2636, Sigma) (store at –20°C).
2. An anion-exchange column Dowex 2 × 8, OH^- form, 20–50 mesh (BioRad) (store at 4°C).
3. 10 mM HEPES buffer, pH 7.4 in endonuclease-free water: 238.3 mg HEPES in 100 mL of sterile endonuclease-free water adjusted to pH 7.4 with a 10 N NaOH and passed through a 0.22 μm sterile syringe filter.
4. *N*-hydroxysuccinimide derivative of the MPEG-propionic acid (MPEG-SPA; Cat # CB0426312, Shearwater, Huntville, AL) (store at –20°C).

5. *N*-hydroxysuccinimide derivative of Boc-His(Boc)-OH (Boc-His(1-Boc)-OSu; Cat # A-1015, BACHEM).
6. *p*-Toluenesulfonic acid, *N*,*N*-diisopropylethylamine, dimethylsulfoxide (DMSO), trifluoroacetic acid (TFA), ethyl acetate.
7. D_2O and NMR tubes.
8. A rotary evaporator.
9. A freeze-dryer.

2.2. Lipids Synthesis

1. Diphenyl phosphite (Cat # D210803, Sigma).
2. 1-Octadecanol (Cat # 258768, Sigma).
3. 3-Aminopropylimidazole (Cat # 272264, Sigma).
4. Histamine.
5. Mannose.
6. Dodecylamine (Cat # 325163, Sigma).
7. 3-Hexadecanoyl-1,3,4-thiadiazoleamine.
8. Chloroform ($CHCl_3$).
9. Carbone tetrachloride (CCl_4).
10. *N*,*N*-diisopropylamine.
11. Diethyl ether.
12. Methyliodide (CH_3I).
13. Methanol (MeOH).
14. Anhydrous dimethylformamide (DMF).
15. Silica gel (pore size 60 Å, 60–100 mesh size silica gel, Cat # 236780, Sigma).
16. Glass column.
17. Pyrex balloons with ground-glass joint.
18. A rotary evaporator.

2.3. mRNA

2.3.1. Preparation and Setup

1. All reactions are prepared with endonuclease-free water, RNase-free tubes and with RNase-free Tips and gloves.
2. All surfaces are cleaned with RNase Decontamination Solution: RNaseZap (Cat # AM9780, AM9782, AM9784, Ambion). Spray the solution onto surfaces to instantly inactivate RNases. Then, rinse twice with RNAse-free water to eliminate all traces of RNase and RNaseZap.
3. An incubator at 37°C.
4. A benchtop microfuge with maximum speed at 16,000 × *g*.

2.3.2. Plasmids

1. pGEM4Z-EGFP-A64, a pDNA encoding the enhanced green fluorescent protein under control of the T7 RNA polymerase promoter (24).

2. pGEM-4Z-MART-1-A64, a pDNA encoding the human MelanA/MART-1 melanoma antigen under the T7 promoter (25).
3. pGEM-4Z-MART-1_LAMP-1-A64, a pDNA encoding the human MelanA/MART-1 melanoma antigen under the T7 promoter (25). This plasmid encodes MART-1 protein carrying the peptide signal and lysosomal sorting signal of human LAMP-1 protein.
4. Dissolve plasmids at 1 μg/μL in TE buffer (10 mM Tris–Cl, pH 7.5 and 1 mM EDTA) in 1.5 mL Eppendorf tubes and store the tubes at −20°C.

2.3.3. DNA Linearization and mRNA Synthesis

1. *Spe*I restriction enzyme (Cat # R01133S, New England Biolabs) at 10 U/μL kit containing NEBuffer 4, BSA (10 μg/mL) and 5 M NH_4OAc.
2. 0.5 M EDTA made in endonuclease-free water.
3. 70% Ethanol.
4. Isopropanol.
5. Microcentrifuge.
6. mMESSAGE mMACHINE® T7 Ultra Kit (Cat #AM1345, Ambion): 1.75 mL Nuclease-free water; 20 μL T7 Enzyme Mix (Buffered 50% glycerol containing RNA polymerase; RNase Inhibitor, and other components); 20 μL 10× T7 Reaction Buffer (salts, buffer, dithiothreitol, and other ingredients); 100 μL T7 2× NTP/ARCA (a neutralized buffered solution containing: 15 mM ATP, 16 mM CTP, 15 mM UTP, 3 mM GTP, 12 mM ARCA); 40 μL T7 GTP (30 mM); 100 μL TURBO DNase 1 (2 U/μL); 10 μL pTRI-Xef 0.5 mg/mL (Control Template); 1 mL Ammonium Acetate Stop Solution (5 M ammonium acetate, 100 mM EDTA); 1.4 mL Lithium Chloride Precipitation Solution (7.5 M lithium chloride, 50 mM EDTA); 1 mL Formaldehyde Load Dye. Store all reagents at −20°C. Water can be kept at room temperature.
7. Poly(A) Tailing Reagents (Cat #AM1350, Ambion): 40 μL *E*-PAP (2 U/μL); 240 μL 5× *E*-PAP Buffer; 100 μL ATP Solution (10 mM); 100 μL 25 mM $MnCl_2$ (store at −20°C).
8. Buffer Phenol:Chloroform 5:1 Acid-Equilibrated pH 4.7 (cat number P1944-100ML, Sigma Aldrich).
9. Phenol-TE-Satured pH 8 (Cat # 77607-250ML, Sigma).

2.3.4. DNA and mRNA Analysis

1. A Nanodrop or an UV spectrophotometer.
2. Electrophoretic mobility shift assay.
3. Sub-Cell electrophoresis cell for submerged horizontal gels.
4. Power supply.
5. 3-(N-morpholino) propanesulfonic acid (MOPS) (Cat # 443832 T, BDH Biochemical VWR).

6. RNA Century™ Marker-Plus (Cat # AM7145, Ambion).
7. 10× MOPS running Buffer: 400 mM MOPS, 100 mM sodium acetate, 10 mM EDTA; Final pH 7.0.
8. Agarose.
9. 37% Formaldehyde.
10. Ethidium bromide 10 mg/mL.
11. An oven at 75°C.
12. An UV light box.

2.4. Preparation of Mannosylated Liposomes (Man-Liposomes)

2.4.1. Preparation and Setup

1. A 5 mL Pyrex balloon with ground-glass joint.
2. A rotary evaporator.
3. Nitrogen gas.
4. An ultrasonic bath equipped with ultrasonic transducer system at 37 kHz (Fisher Scientific).
5. Dialysis Tubing Cellulose membrane of 33 mm × 21 mm size with a molecular weight cutting out at 12.4 kDa (Cat #D9652, Sigma Aldrich).
6. 1.5 mL Eppendorf tubes.
7. 96-Well polystyrene crystal microtiter plates.
8. A microplate spectrofluorometer.
9. A microplate spectrophotometer.
10. An oven at 90°C.
11. 10 mM HEPES buffer, pH 7.4.
12. A Nile Red (Cat # 19123, Sigma) solution (1 μM in methanol).
13. Resorcinol (6 mg/mL) (Cat # 107593, Merck).
14. Mannose.
15. Pristane.
16. 75% Sulfuric acid solution: 100 mL sulfuric acid (analytical grade, 95%; $d = 1.834$), 24 mL of distilled water. Keep at room temperature in the dark for up to 3 weeks.
17. 5% Sodium nitrite in water.
18. 0.5% Sulfanilic acid in 5% HCl.
19. 10% Sodium carbonate in water.

2.4.2. Size ζ Potential Measurements

1. ZetaSizer 3000 (Malvern Instruments).
2. Polystyrene polymer of 200 ± 5 nm in diameter (Duke Scientific Corps Palo Alto, CA, USA).
3. DTS 5050 standard beads of −50 mV from Malvern.
4. 10 × 10 × 45 mm Polystyrene Spectrophotometry Cuvettes.
5. 10 mM HEPES buffer pH 7.4.

2.5. Cells and Cell Culture

1. Murine melanoma B16/F10 cells (ATCC CRL 6475).
2. Murine dendritic DC2.4 cells (26).
3. PBS.
4. 0.5 M EDTA, pH 7.2.
5. Trypsin/EDTA solution: dilute 10 mL trypsin/EDTA solution (Cat # R-001-100, Life Technologies) in 100 mL PBS.
6. DMEM complete medium: Dulbecco's Modified Eagle's Medium containing 10% heat-inactivated fetal bovine serum (FCS), 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 U/mL streptomycin.
7. Cell growth: Seed 1×10^6 cells in a 75 cm^2 culture flask with 20 mL complete culture medium, and incubate at 37°C in a humidified atmosphere in the presence of 5% CO_2.
8. Cell passage: Harvest the cells at confluence: aspirate medium, wash cells with 10 mL PBS, treat for 10 min at 37°C with 10 mL trypsin/EDTA solution, add 10 mL complete culture medium to block trypsin, collect cell suspension, and spin in a 50 mL sterile centrifuge tube for 15 min at 300 × *g* in a chilled Benchtop Centrifuge.

2.6. In Vitro mRNA Transfection

1. 24-Well culture plates.
2. 12 × 75-mm FACS Tube Acquisition.
3. Flow cytometer (LSR1, Becton Dickinson).
4. Chilled Benchtop Centrifuge.
5. Cold methanol/PBS mixture (9:1; v/v).
6. PBS containing 1% BSA.
7. PE-labeled anti-Melan-A antibodies (Cat # SC-20032 PE, Tebu-bio, Le Perray en Yvelines, France).

2.7. Splenocytes Preparation

1. Petri dishes with 10 mL cold sterile PBS.
2. Scalpels.
3. Nylon membrane: 40 μm nylon mesh (Cell Strainer) (Cat #35-2340; Becton Dickinson).
4. Syringes with 25-G needles.
5. Sterile nylon membrane, Histopaque 1083 (Cat # 1083-1, Sigma).
6. 15 mL sterile polystyrene conical centrifuge tube.
7. Chilled Benchtop Centrifuge.

2.8. CTL Response

1. CytoTox 96 Non-Radioactive Cytotoxicity Assay (Cat # G1780, Promega).
2. Mitomycin C.

3. 96-well U-shaped microtiter plates.
4. A 75 cm^2 culture flask of confluent B16/F10 cells.
5. Spleens from immunized and nonimmunized mice 7 days after the last immunization.
6. RPMI complete medium: RPMI-1640 medium containing 10% FCS, 2 mMl-glutamine, 1 mM sodium pyruvate, 1 mM amino acids, 100 U/mL penicillin/streptomycin, and 50 U/mL mouse recombinant IL-2.

2.9. CTLs: IFN-γ Secretion

1. Spleens from immunized and nonimmunized mice 7 days after the last immunization.
2. A 75 cm^2 culture flask of confluent B16/F10 cells.
3. RPMI complete medium.
4. PSE: PBS containing 0.5% BSA and 2 mM EDTA.
5. 12 × 75-mm FACS Tube Acquisition (BD Biosciences).
6. Mouse IFN-γ secretion assays-detection kit (Cat # 130-090-516, Miltenyi Biotec, Paris, France).
7. FITC-labeled anti-mouse CD4 antibodies (Cat # 130-091-608, clone GK1.5, Miltenyi Biotec).
8. FITC-labeled anti-mouse CD8 antibodies (Cat # 130-091-605, clone 53-6.7, BD Pharmingen, San Diego, CA).
9. PE-labeled anti-mouse IFN-γ antibodies (Cat # 130-092-346, Miltenyi Biotec).
10. IFN-γ Catch Reagent (Cat # 130-090-516, Miltenyi Biotec).
11. A flow cytometer.

3. Methods

3.1. PEG-HpK Synthesis

3.1.1. HpK Synthesis

1. Dissolve 1 g of poly-l-lysine in 200 mL water and pass the solution through an anion-exchange column (Dowex 2 × 8, -OH form, 20–50 mesh) (see Note 1).
2. Neutralize the eluate with a 10% *p*-toluenesulfonic acid solution water and freezed-dry the solution.
3. Dissolve polylysine *p*-toluene sulfonate salt (200 mg; 3.44 μmol) in 12 mL DMSO in a 100 mL pyrex balloon with ground-glass joint and add 55 μL *N*,*N*-diisopropylethylamine.
4. Add under agitation 175 mg of Boc-His(1-Boc)-OSu dissolved in 3 mL DMSO. Keep the reaction for 24 h at room temperature under agitation.

5. Add 20 mL of a gold solution of H_2O/TFA (1:1; v/v) for Boc deprotection. Keep the reaction overnight at room temperature under agitation.
6. Evaporate TFA and water under reduced pressure.
7. Add the solution into 20 volumes of ethyl acetate to precipitate the polymer.
8. Collect the precipitate by centrifugation at 1,800×*g* for 15 min.
9. Solubilize the precipitate in 20 mL distilled water in a 100 mL pyrex balloon with ground-glass joint and freeze-dry.
10. Solubilize 5 mg of polymer powder in 0.7 mL D_2O, put the solution in an NMR tube and record the^{1}H-NMR spectrum at 500 MHz.
11. Determine the number of His residues *per* polymer molecule from the ^{1}H-NMR spectrum (see Note 2).

3.1.2. PEG-HpK Synthesis

1. Dissolve 250 mg of Histidylated polylysine (HpK) (3.5 µmol) in 3 mL DMSO in a 100 mL pyrex balloon with ground-glass joint and add 116 µL of *N*,*N*-diisopropylamine (850 µmol).
2. Under agitation, add 29 mg (5 µmol) of MPEG-SPA dissolved in 0.5 mL DMSO.
3. After 4 h at 20°C, add the solution into 20 volumes of ethyl acetate to precipitate the polymer.
4. Collect the precipitate by centrifugation at 1,800×*g* for 15 min.
5. Solubilize the precipitate in distilled water and freeze-dry.
6. Solubilize 5 mg of polymer powder in 0.7 mL D_2O, put the solution in an NMR tube and record the ^{1}H-NMR spectrum at 500 MHz.
7. Determine the number of PEG molecule bound *per* polymer molecule from the ^{1}H NMR spectrum (see Note 3).
8. Prepare aliquots of 1.5 mg PEGylated Histidylated Polylysine (PEG-HpK) in 1 mL sterile 10 mM HEPES buffer, pH 7.4 in endonuclease-free water in 1.5 mL Eppendorf tube and store at –20°C.

3.2. Lipids Synthesis

3.2.1. O,O-Dioleyl-N-[3N-(N-methylimidazolium iodide)propylene] phosphoramidate (Lipid 1)

1. Mix 234 g (1 mmol) of diphenyl phosphate and 541 g (2 mmol) of 1-octadecanol in a 100 mL pyrex balloon with ground-glass joint and heat for 3 h at 120°C.
2. Eliminate the phenol formed during the reaction by distillation under vacuum. The reaction is quantitative and leads to the dioleylphosphite compound.

3. Mix 2.91 g (5 mmol) of dioleylphosphite and 597 μL (5 mmol) of 3-aminopropylimidazole in 20 mL $CHCl_3$ in a 100 mL pyrex balloon with ground-glass joint.
4. Add 530 μL (5.5 mmol) CCl_4 under nitrogen at 0°C.
5. Add slowly 870 μL (5 mmol) of *N*,*N*-diisopropylamine and stir the mixture for 2 h at 0°C and then overnight at 20°C.
6. Evaporate the solvent under reduced pressure.
7. Dissolve the product in diethyl ether.
8. Remove the ammonium salts by filtration, and concentrate the residue product under reduced pressure.
9. Dissolve the residue in 3 mL of CH_3I, and stir the solution overnight at 20°C.
10. Evaporate the volatiles.
11. Dissolve the product in 5 mL $CHCl_3$, and purify the lipid by silica gel chromatography using a $CHCl_3$/MeOH (90:10; v/v) solvent.
12. Lipid **1** is obtained as orange oil after concentration.
13. Perform ^{1}H-NMR and mass spectra and compare to data given in ref. (21).

*3.2.2. O,O-Dioleyl-N-histamine phosphoramidate (Lipid **2**)*

Lipid **2** is synthesized as lipid **1** using dioleylphosphite and histamine. Perform ^{1}H-NMR and mass spectra and compare to data given in ref. (22).

3.2.3. β-d-Mannopyranosyl-N-dodecylhexadecanamide (Man-lipid)

Briefly, Man-lipid is obtained by reductive amination of mannose with dodecylamine in DMF followed by N-acylation with 3-hexadecanoyl-1,3,4-thiadiazoleamine in DMF.

3.2.4. Lipids Stock Solutions

Prepare 5.4 mM lipid **1**, 5.4 mM lipid **2** and 5.4 mM Man-lipid in ethanol. Store at −20°C.

3.3. Preparation of Mannosylated Liposomes (Man-liposomes)

3.3.1. Preparation

1. Mix 1 mL of 5.4 mM lipid **1** in ethanol, 1 mL of 5.4 mM lipid **2** in ethanol, and 0.15 mL of 5.4 mM Man-lipid in a 5 mL Pyrex balloon with ground-glass.
2. Evaporate ethanol with a rotary evaporator under reduced pressure until formation of a film. At the end, the atmospheric pressure is restored by the introduction of nitrogen gas.
3. Add 1 mL of sterile endonuclease-free 10 mM HEPES buffer, pH 7.4 and hydrate the film for 12 h at 4°C.
4. Vortex vigorously the solution and sonicate it for 15 min at 20°C in an ultrasonic bath at 37 kHz.
5. Put the liposomes in a dialysis bag and dialyse at 4°C for 6 h and then overnight against 500 mL of sterile endonuclease-free 10 mM HEPES buffer, pH 7.4 (see Note 4).

6. Collect Man-liposomes in 1.5 mL Eppendorf tube.
7. Adjust the Man-liposomes concentration to 5.4 mM with sterile endonuclease-free 10 mM HEPES buffer, pH 7.4 and store the solution at −4°C.

3.3.2. Lipid Assay

After dialysis, the lipid concentration is determined with Nile Red.

1. Add 10 μL of liposomes in 70 μL of 10 mM HEPES, pH 7.4 in 96-well U-shaped microtiter plates and mix with 20 μL of a Nile Red solution (1 μM in methanol).
2. Agitate the mixture for 5 min at room temperature on an agitating plate.
3. Measure the fluorescence intensity of each well at 620 nm upon excitation at 531 nm with a microplate spectrofluorometer.

3.3.3. Sugar Assay

After dialysis, the amount of Man-lipid incorporated in liposomes is determined using the colorimetric resorcinol/sulfuric assay (27).

1. Distribute 20 μL of liposomes sample at various dilutions in a U-shaped well of a 96-well microtiter plate and add 20 μL of 6 mg/mL resorcinol, 100 μL of 75% sulfuric acid, and 50 μL of pristane.
2. Distribute 20 μL of mannose at various concentrations ranged from 0 to 100 μg/mL in order to generate a mannose standard curve to determine the amount of incorporated Man-lipid.
3. Homogenize the solutions by shaking the plate with a vortex.
4. Heat the plate at 90°C in an oven for 30 min.
5. Keep the plate at room temperature for 30 min in the dark.
6. Measure the absorbance of each well at 430 nm with the microplate spectrophotometer.
7. Calculate the amount of incorporated Man-lipid.

3.3.4. Histidine Assay

After dialysis, the amount of imidazole or histidine in liposomes is determined by the Pauly colorimetric assay (28).

1. Distribute 20 μL of liposomes samples to 100 μL of 10 mM HEPES buffer, pH 7.4 in a 96-well microtiter plate.
2. Distribute 20 μL of various concentrations of histidine ranged from 0 to 100 μg/mL to 100 μL of 10 mM HEPES buffer, pH 7.4 in a 96-well microtiter plate to generate a histidine standard curve to determine the amount of lipid containing histidine or imidazole in liposomes.

3. Add 100 μL of a v/v solution of (5% sodium nitrite in water/0.5% sulfanilic acid in 5% HCl) and 100 μL of 10% sodium carbonate in water.
4. Mix and allow the reaction during 30 min at room temperature in the dark on an agitating plate.
5. Read the absorbance of each well at 490 nm with the microplate spectrophotometer.
6. Calculate the amount of lipid containing histidine or imidazole *per* liposomes.

3.4. In Vitro Transcribed mRNA

pGEM4Z-EGFP-A64, pGEM-4Z-MART-1-A64, and pGEM-4Z-MART-1_LAMP-1-A64 plasmids are constructed to contain a unique *Spe*I endonuclease cleavage site (see Note 5).

3.4.1. Preparation of Template DNA: Plasmid DNA Linearization

1. Put in a sterile Eppendorf tube in the following order: 37 μL sterile deionized water, 5 μL NEBuffer 4, 5 μL BSA, 2 μL of plasmid DNA.
2. Mix by pipetting and add 1 μL *Spe*I.
3. Incubate at 37°C for 2 h.
4. Stop the reaction by heating at 80°C for 20 min.
5. The digestion is completed by adding in the following order: 2.5 μL of 0.5 M EDTA, 5 μL of 5 M NH_4OAc, and 100 μL ethanol.
6. Mix and chill at –20°C for at least 15 min.
7. Pellet the DNA in a microcentrifuge for 15 min at top speed (16,000 × *g*).
8. Remove the supernatant.
9. Spin again the tube for a few seconds.
10. Remove the supernatant with a very fine-tipped pipette.
11. Solubilize the pellet in nuclease-free water at a DNA concentration of 0.5–1 μg/μL using the UV light absorbance at 1 U $A_{260} = 50$ μg/mL.

3.4.2. Synthesis of Capped mRNA-A64

The following protocol can provide 100–150 μg mRNA.

1. Thaw the frozen reagents of the mMESSAGE mMACHINE® T7 Ultra Kit.
   (a) Place the T7 Enzyme Mix on ice.
   (b) Vortex the 10× T7 Reaction Buffer and the T7 2× NTP/ARCA (see Note 6) until they are completely in solution.
   (c) Once thawed, store T7 2× NTP/ARCA on ice, but keep the 10× T7 Reaction Buffer at room temperature while assembling the reaction.

   (d) Microfuge all reagents briefly before opening tubes to prevent loss and/or contamination of material that may be present around the rim of the tube.

2. Assemble transcription reaction at room temp (see Note 7). Add in the following order in a 1.5 mL RNase-free tubes.
   (a) 100 μL Nuclease-free Water,
   (b) 50 μL T7 2× NTP/ARCA,
   (c) 10 μL 10× T7 Reaction Buffer,
   (d) 10 μg linear template DNA
   (e) 10 μL T7 Enzyme Mix.
3. Gently flick the tube or pipette the mixture up and down gently.
4. Microfuge tube briefly to collect the reaction mixture at the bottom of the tube.
5. Incubate at 37°C overnight (see Note 8).
6. Add 5 μL TURBO DNase, mix well and incubate 15 min at 37°C to remove the template DNA.

3.4.3. Poly(A) Tailing Procedure (see Note 9)

1. Reserve 2.5 μL of the reaction mixture. This minus-enzyme control will be run on a gel next to the tailed RNA at the end of the experiment.
2. Add to the 100 μL mMESSAGE mMACHINE T7 Ultra reaction in the following order:
   (a) 180 μL nuclease-free water,
   (b) 100 μL 5× E-PAP Buffer,
   (c) 50 μL 25 mM $MnCl_2$,
   (d) 50 μL ATP Solution.
3. Add 20 μL E-PAP, mix gently. The final reaction volume is 500 μL.
4. Incubate at 37°C for 2 h.
5. Place the reaction on ice.

3.4.4. Recovery of the mRNA

1. Add 50 μL Ammonium Acetate Stop Solution to the 500 μL poly(A) Tailing reaction, and mix thoroughly.
2. Extract with 500 μL of phenol:chloroform 5:1 acid-equilibrated pH 4.7.
3. Mix by vortexing for few seconds.
4. Centrifuge for 5 min at maximum speed (16,000 × *g*).
5. Recover the aqueous phase and transfer to new 1.5 mL RNase-free tube.

6. Extract the aqueous phase with an equal volume of Phenol-TE-Satured pH 8, mix by vortexing for few seconds.
7. Centrifuge for 5 min at maximum speed (16,000 ×*g*).
8. Recover the aqueous phase and transfer to new 1.5 mL RNase-free tube.
9. Add 1 volume of isopropanol and mix well to precipitate the RNA.
10. Chill the mixture for at least 15 min at –20°C.
11. Centrifuge at 4°C for 15 min at maximum speed (16,000 ×*g*) to pellet the RNA.
12. Carefully remove the supernatant.
13. Wash the pellet once with ~1 mL 70% ethanol.
14. Centrifuge the solution at 4°C for 15 min at maximum speed (16,000 ×*g*) to pellet the RNA.
15. Carefully remove the supernatant
16. Dissolve mRNA in nuclease-free water.
17. Store mRNA at –80°C in small aliquots in polypropylene cryotubes.

3.4.5. mRNA Quantitation

1. Read the absorbance at 260 nm (A_{260}) and at 280 nm (A_{280}) of a 1 μL of mRNA with the nanodrop.
2. Check the purity of mRNA from the A_{260}/A_{280} ratio. For high purity this ratio must be equal to 2.0. A ratio value of 1.9–2.1 is accepted.
3. Calculate the amount of single-stranded RNA using 1 A_{260} unit = 40 μg/mL.

3.4.6. mRNA Quality: Denaturing Agarose Gel Electrophoresis

1. Prepare a 1% agarose gel (see Note 10). For 100 mL of gel, dissolve 1 or 2.5 g agarose in 72 mL water in a microwave device.
2. Cool the solution to 60°C.
3. Add 10 mL 10× MOPS running buffer, and 18 mL 37% formaldehyde (12.3 M).
4. Pour the gel and allow it to set. The wells should be large enough to accommodate at least 60 μL. Remove the comb, and place the gel in the gel tank. Cover with a 1× MOPS running buffer.
5. Preparation of the RNA samples: add 7.5 μL Formaldehyde Load Dye containing 50 μg/mL ethidium bromide to 2.5 μL aliquot of each tailing reaction, the corresponding minus-poly(A) Tailing control and an RNA size marker.
6. Denature mRNA by heating at 65°C for 10 min and then put into ice for 10 min.

7. Load the samples.
8. Run the gel in 1× TBE buffer at ~5 V/cm until the bromophenol blue dye is near the bottom of the gel.
9. Examine the gel on an UV light box (see Note 11).

3.5. MART1 mRNA Lipopolyplexes (MART1 Man-LPR)

Preparation of a dose for three mice (see Note 12):

1. Add 50 μl (50 μg) MART1 mRNA in 1.5 mL Eppendorf tube containing 160 μL sterile 10 mM HEPES buffer, pH 7.4 in endonuclease-free water.
2. Add 100 μl (150 μg) of the PEG-HpK solution.
3. Mix for 4 s and keep for 30 min at 20°C.
4. Add 50 μL Man-liposomes (100 μg in sterile 10 mM HEPES buffer, pH 7.4 in endonuclease-free water) in the MART1 mRNA polyplexes solution (see Note 13).
5. Mix gently by up-down pipetting and keep for 15 min at 20°C.
6. Add 40 μL of a 50% sucrose solution in endonuclease-free water (see Note 14).

3.6. MART1_LAMP1 mRNA Lipopolyplexes (MART1_LAMP1 Man-LPR) (see Note 15)

Preparation of a Dose for three mice (see Note 12):

1. Add 50 μl (50 μg) MART1_LAMP1 mRNA in 1.5 mL Eppendorf tube containing 160 μL sterile 10 mM HEPES buffer, pH 7.4 in endonuclease-free water.
2. Add 100 μl (150 μg) of the PEG-HpK solution.
3. Mix for 4 s and keep for 30 min at 20°C.
4. Add 50 μL Man-liposomes (100 μg in sterile 10 mM HEPES buffer, pH 7.4 in endonuclease-free water) in the MART1_LAMP1 mRNA polyplexes solution (see Note 13).
5. Mix gently by up-down pipetting and keep for 15 min at 20°C.
6. Add 40 μL of a 50% sucrose solution in endonuclease-free water.

3.7. Electrophoretic Mobility Shift Assay of mRNA and LPR

Check the quality of mRNA and LPR formation by electrophoresis in a 1% agarose-formaldehyde gel as described in Subheading 3.2, step 6. As shown in Fig. 4, EGFP mRNA migrates as a single band at 915 nt. When formulated with LPR or Man-LPR, all the mRNA is complexed with the polymer and liposome and does not migrate.

3.8. Lipopolyplex Size Measurements

1. Set up the following parameters on the ZetaSizer: viscosity, 0.891 cP; dielectric constant, 79; temperature, 25°C; F(Ka), 1.50 (Smoluchowsky); maximum voltage of the current, 15 V.

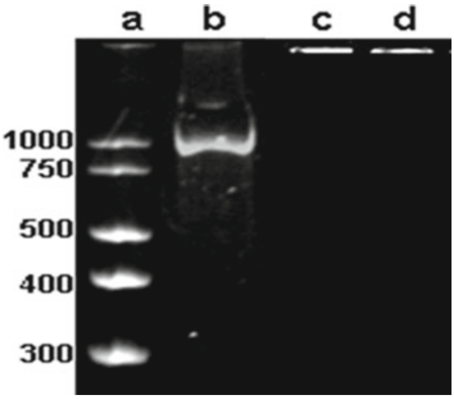


Fig. 4. Electrophoretic mobility shift assay of mRNA and LPR. LPR and Man-LPR are formed with EGFP mRNA. mRNA (1 μg) and LPR (1 μg mRNA) are loaded in a 1% agarose–formaldehyde gel and electrophoresis is run for 1 h at 90 V in MOPS. Lane a: RNA Century™ Marker-Plus; lane b: EGFP mRNA (915 nt); lane c: LPR; lane d: Man-LPR. RNA is stained with ethidium bromide.

2. To calibrate the size measurement, put 200 ± 5 nm polystyrene polymer into a polystyrene cuvette containing 1.4 mL of 10 mM HEPES buffer pH 7.4. Insert the cuvette in the size measurement place of the apparatus.
3. Measure the size ten times under the automatic mode.
4. Then, add 20 μL of Man-LPR loaded with 5 μg mRNA into a polystyrene cuvette containing 1.4 mL of 10 mM HEPES buffer pH 7.4. Insert the cuvette in the size measurement place of the apparatus.
5. Measure the size of lipopolyplexes ten times under the automatic mode.

3.9. Lipopolyplex ζ Potential Measurements

1. Set the following parameters on the ZetaSizer: viscosity, 0.891 cP; dielectric constant, 79; temperature, 25°C; F(Ka), 1.50 (Smoluchowsky); maximum voltage of the current, 15 V.
2. To calibrate the ζ potential measurement, put 0.7 mL of DTS 5050 standard beads of −50 mV into a polystyrene cuvette and insert it in the aqueous dip cell of the apparatus and the electrodes on the dip cell.
3. Measure the ζ potential ten times with the zero field correction.
4. Then put 10 μL of Man-LPR loaded with 2.5 μg mRNA into a polystyrene cuvette containing 0.7 mL of 10 mM HEPES

buffer pH 7.4. Insert the cuvette in the aqueous dip cell of the apparatus and the electrodes on dip cell.

5. Measure the ζ potential ten times with the zero field correction.

3.10. In Vitro EGFP mRNA Transfection

1. Two days before transfection, seed 10^5 DC2.4 cells in 24-well culture plates.
2. At the time of transfection, remove the culture medium and wash the cells with 1 mL serum-free medium.
3. Add 0.5 mL of Man-LPR loaded with 5 μg EGFP mRNA to the cells and incubate for 4 h at 37°C in CO_2 incubator.
4. Remove the transfection medium, add 1 mL fresh complete culture medium and culture the cells for 22 h.
5. Remove the culture medium and add 0.5 mL of trypsin/EDTA solution for 5 min at 37°C to harvest the cells.
6. Put the cell suspension in 12 × 75-mm FACS Tube Acquisition (BD Biosciences), add 3 mL PBS, centrifuge the tube for 10 min at 300 × *g*.
7. Remove the supernatant, add 3 mL PBS, centrifuge the tube for 10 min at 300 × *g*, remove the supernatant, and suspend the cell pellet in 0.5 mL PBS.
8. Measure the cell-associated EGFP fluorescence intensity with a BDLSR flow cytometer at $\lambda_{ex} = 488$ nm and $\lambda_{em} = 530 \pm 30$ nm.

3.11. In Vitro MART1 mRNA Transfection

1. Two days before transfection, seed 10^5 DC2.4 cells in 24-well culture plates
2. At the time of transfection, remove the culture medium, add 1 mL serum-free medium for 5 min, and remove it.
3. Add 0.5 mL of Man-LPR loaded with 5 μg MART1 mRNA and incubate the cells for 4 h at 37°C.
4. Remove the transfection medium, add 1 mL fresh culture medium, and incubate the cells for 24 h at 37°C in a CO_2 incubator.
5. Remove the culture medium, add 0.5 mL of PBS containing 0.2 mM EDTA, incubate 5 min at 37°C, collect the cells in 12 × 75-mm FACS Tube Acquisition (BD Biosciences), add 3 mL PBS, and centrifuge the tubes at 300 × *g*.
6. Add 1 mL of a cold methanol/PBS mixture (9:1; v/v) on the cell pellet and incubate for 30 min at room temperature.
7. Wash the cells two times with 3 mL PBS by centrifugation at 300 × *g*.
8. Add 500 μL PBS containing 1% BSA and incubate the cells for 30 min at room temperature.

9. Wash the cells two times with 3 mL PBS containing 1% BSA by centrifugation at 300 × *g*.
10. Add 100 μL PBS containing 1% BSA and PE-labeled anti-Melan-A antibodies (Tebu-bio, Le Perray en Yvelines, France) diluted ten times on the cell pellet and incubate for 20 min at room temperature.
11. Wash the cells two times with 3 mL PBS containing 1% BSA by centrifugation at 300 × *g*.
12. Measure the cell-associated fluorescence intensity with a BDLSR flow cytometer at $\lambda_{ex} = 488$ nm and $\lambda_{em} = 585 \pm 30$ nm.

3.12. Vaccination Schedule

For vaccination with Man-LPR, we recommend intravenous injections (see Note 16).

1. Prepare the vaccine solution by mixing in 1.5 mL Eppendorf tube 400 μL of MART1 Man-LPR with 400 μL of MART1_LAMP1 Man-LPR.
2. At day 0, inject three mice in the tail vein with 200 μL vaccine solution.
3. Repeat injections on days 7 (see Note 17).

3.13. Primary Tumor Challenge

1. On day 14, perform intradermal injection of 100 μL PBS containing 10^5 B16F10 cells in mice.
2. Measure the tumor volume three times a week.
3. Calculate the tumor volume (see Note 18).
4. For ethical reasons, euthanize animals that have developed large tumors (see Note 19).

3.14. Splenocytes Preparation

1. Harvest the spleens of mice, put them in a Petri dish with 10 mL cold sterile PBS and cut in small pieces with a scalpel.
2. Take the cell suspension with a syringe with a 25-G needle, pass the cell suspension through a sterile nylon membrane and collect the cells in a 15 mL conic centrifuge tube.
3. Centrifuge the tube for 10 min at 300 × *g* at room temperature.
4. Suspend the cells pellet in 1.5 mL PBS and deposit the cell suspension on the surface of 1.5 mL Histopaque 1083 in a 15 mL conic centrifuge tube at room temperature.
5. Centrifuge the tube for 30 min at 400 × *g* at room temperature.
6. Collect the liquid interface gently with a pipette, put it in 3 mL PBS in a 15 mL sterile conic centrifuge tube and centrifuge the tube for 10 min at 300 × *g* at room temperature.

3.15. CTLs: Cytotoxic Activity

The presence of CTL is measured by their capacity to lyse tumor cells. For his purpose, the lactate dehydrogenase (LDH) colorimetric assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega) is used (see Notes 20 and 21).

1. Two days before the experiment, seed 2.5×10^5 B16/F10 cells in 5 mL DMEM complete medium in a 6-well culture plate and incubate at 37°C in CO_2 incubator.
2. Then, add 50 μg/mL of mitomycin C in each well and incubate the culture plate for 1 h at 37°C in CO_2 incubator.
3. The day of the experiment, collect spleens from immunized and nonimmunized mice 7 days after the last immunization.
4. Prepare single cell suspensions from spleen as described in Subheading 3.14.
5. Remove the culture medium of B16/F10 cells incubated in the presence of mitomycin C.
6. Add 5 mL RPMI complete medium.
7. Distribute 10^7 freshly isolated splenocytes in the 6-well culture plate containing mitomycin C-inactivated B16/F10 cells for splenocytes stimulation and incubate the plate for 3 days at 37°C in CO_2 incubator.
8. *S wells*: Distribute in triplicate serial dilutions of stimulated splenocytes (effector cells) in 5×10^3 untreated B16/F10 cells (target cells) in 100 μL medium in a 96-U shaped microtiter plate.
9. E_{min} *wells*: Distribute in triplicate serial dilutions of stimulated splenocytes (effector cells) in 100 μL medium in a 96-U shaped microtiter plate without target cells.
10. E_{max} *wells*: Distribute in triplicate serial dilutions of stimulated splenocytes (effector cells) in 100 μL medium plus 10 μL of Triton X-100 lysis solution in a 96-U shaped microtiter plate without target cells.
11. T_{min} *wells*: Distribute in triplicate 5×10^3 untreated B16/F10 cells (target cells) in 100 μL medium in a 96-U shaped microtiter plate.
12. T_{max} *wells*: Distribute in triplicate 5×10^3 untreated B16/F10 cells (target cells) in 100 μL medium plus 10 μL of Triton X-100 lysis solution in a 96-U shaped microtiter plate.
13. Incubate the plates for 5 h at 37°C in CO_2 incubator.
14. Collect and distribute 50 μL of supernatants in each well of a 96-well microtiter plate.
15. Add 50 μL of reconstituted substrates.
16. Incubate the plate for 30 min at room temperature in the dark.

17. Measure the absorbance at 490 nm with a microplate spectrophotometer.
18. Calculate the percentage of cell lysis from the formula: $100 \times (S - T_{\min} - E_{\min}) / (T_{\max} - E_{\max})$.

3.16. CTLs: IFN-γ Secretion (see Note 22)

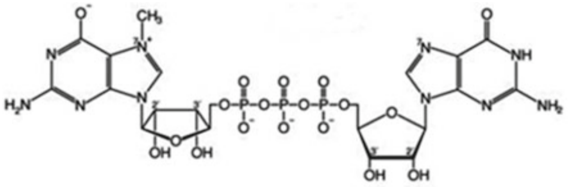
1. Two days before the experiment, seed 2.5×10^5 B16/F10 cells in 5 mL DMEM complete medium in a 6-well culture plate and incubate at 37°C in CO_2 incubator.
2. The day of the experiment, collect spleens from immunized and nonimmunized mice 7 days after the last immunization.
3. Prepare single cell suspensions from spleen as described in Subheading 3.14.
4. Cocultivate freshly isolated spleen cells (10^7 cells) with 10^6 B16/F10 cells for 20 h at 37°C in 6-well culture plates with 5 mL RPMI complete medium.
5. Collect splenocytes (non-adherent cells) and wash them by centrifugation ($300 \times g$) with PSE.
6. Suspend the cells in 2 mL cold RPMI complete medium. Count the splenocytes and make the suspension at 10^7 splenocytes *per* mL.
7. Distribute the splenocytes (10^6 cells in 100 μL) in 12 × 75-mm FACS Tube Acquisition, add 10 μL of IFN-γ Catch Reagent and incubate for 10 min at 4°C.
8. Add 1 mL culture medium.
9. Incubate the cells for 45 min at 37°C.
10. Wash the cells several times with PSE by centrifugation ($300 \times g$).
11. Add in different tubes, 50 μL PSE containing FITC-labeled anti-mouse CD4 antibodies (diluted ten times) or FITC-labeled anti-mouse CD8 antibodies (diluted 100 times) and PE-labeled anti-mouse IFN-γ antibodies (diluted ten times). Incubate the tubes for 10 min at 4°C.
12. Measure the cell fluorescence intensity by flow cytometry. Gate the lymphocyte population with the Forward-Scatter and Side-Scatter, and measure the fluorescence intensity of 10,000 cells at 520 nm (FL-1 channel) and 585 nm (FL-2 channel) upon excitation at 488 nm (see Note 23).

4. Notes

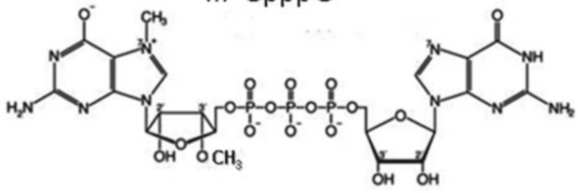
1. This protocol aims to remove bromide ions which can induce cytotoxicity. Moreover, polylysine *p*-toluene sulfonate salt is highly soluble in dimethylsulfoxide, a better solvent than an

aqueous buffer to modify the lysyl side chains with *N*-hydroxysuccinimide derivatives of histidine and PEG residues.

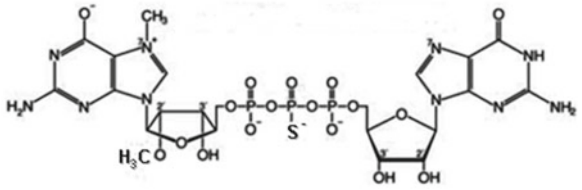
2. The number of His residues *per* polymer molecule is determined from the ^{1}H-NMR spectrum in D_2O according to $x = 6(h_{8.7}/h_{\text{lys}})$.dp, where $h_{8.7}$ is the value of the integration of the signal at 8.7 ppm corresponding to the proton of the imidazole ring, h_{lys} is the range 1.3–1.9 ppm corresponding to the 6 methylene protons of lysine residues, and dP is the degree of polylysine polymerization.
3. The number of PEG molecule bound *per* polymer molecule is determined from the ^{1}H NMR spectrum in D_2O using peak intensity ratio of the methylene protons at 3.5 ppm of PEG (~454 methylene protons *per* PEG 5000 molecule) to those of the lysyl side chain at 1.2–1.3 ppm (6 methylene protons *per* HpK molecule).
4. Liposomes are dialysed in order to eliminate any cytotoxicity. We have reported that Man-liposomes are devoid of any cytotoxicity upon their dialysis (20). The presence of glycolipid molecules in liposomes did not strongly affect their size which remained comprised between 125 and 200 nm. Dialysis reduced the zeta potential of Man-liposomes.
5. pGEM4Z-EGFP-A64, pGEM4Z-MART-1-A64, and pGEM4Z-MART-1_LAMP-1-A64. These vectors contain the entire coding sequence for EGFP, MART-1, and MART-1_LAMP-1, respectively. The coding sequence is followed by 64 A-T bp and by a unique *Spe*I restriction site.
6. The use of 3′-*O*-methyl-m7$^{5\prime}$Gppp5′G, an anti-reverse cap analogue (ARCA), avoids the cap incorporation in the reverse orientation (29) (Fig. 5). Consequently, in vitro ARCA-capped transcripts (ARCA-mRNA) were found to exhibit high translation efficiency. We have optimized a luciferase mRNA construct for allowing a high level of protein upon in vitro transfection of mouse and human dendritic cells (30). We have shown that luciferase activity was 20-fold higher when the luciferase mRNA was capped with 3′-*O*-methyl-m7$^{5\prime}$Gppp$^{5\prime}$G (termed anti-reverse cap analogue; ARCA) than with the classical m7$^{5\prime}$Gppp$^{5\prime}$G. Note that β-S-ARCA derivative containing a phosphorothioate modification was found to further increase mRNA stability and the translational efficiency (31, 32) (Fig. 5).
7. The spermidine in the 10× T7 Reaction Buffer can coprecipitate the template DNA if the reaction is assembled on ice.
8. An overnight incubation allows doubling the mRNA quantity compared to the 2 h incubation recommended by the manufacturers.



m^{7}GpppG



$m_2^{7,3'\text{-}O}$GpppG (ARCA)



$m_2^{7,2'\text{-}O}$Gpp$_S$pG (β-S-ARCA)

Fig. 5. Functional cap analogs proposed to date.

9. We also showed that the addition of a Poly(A) tail of 100 instead of 64 adenosines in *cis* increased by 35-fold the protein level. Finally, ARCA-Luc-mRNA-A100 construct was 700-fold more efficient than the CAP-Luc-mRNA-A64 one. Such mRNA construct was also successful to obtain high level of MART-1 tumor antigen in DC in vitro and in vivo (15, 30).

10. A 2.5% agarose gel is used for mRNA Transcript size ≤500 bases, while a 1% agarose gel for mRNA Transcript size >500 bases.

11. The quality of mRNA preparation is checked by agarose-formaldehyde gel electrophoresis. Figure 16.4 lane b is a typical mRNA band corresponding to a good quality of mRNA. First, there is a single band corresponding to the expected size according to the RNA size markers. Second, there is no smirs in the mRNA lane which is the case when the mRNA preparation is not good and the mRNA is degraded or contains incomplete transcriptions. When it is the case, the preparation should be run again.

12. In fact this preparation takes into account the losses incurred during the intravenous injections. Thus, for three mice it is better to make for four mice.

13. Before use, the Man-liposomes solution stocked at 4°C is sonicated for 10 min at 20°C in the ultrasonic bath and then cooled on ice.
14. Since mRNA Man-LPR is not prepared at physiological salt concentration, adding 40 μL of a 50% sucrose solution makes the solution to be at 5% sucrose which is a isotonic solution used as vehicle and diluent for pharmaceutical products compatible to administer intravenous parenteral.
15. When mice were injected with a mixture of MART1 mRNA LPR and sig-MART-1_LAMP-1 mRNA LPR, the antitumor response was significantly higher than that induced by MART1 mRNA LPR alone. In contrast, the injection of sig-MART-1_LAMP-1 mRNA LPR alone did not protect mice against B16 melanoma. The higher protective effect obtained with the combination of both MART1- and sig-MART-1_LAMP-1 mRNA suggests that the immune response is improved by enhancement of the CD4$^+$ T-cells activation. This synergic effect is not yet explained. The pGEM4Z/sig-MART-1LAMP-1-A64 mRNA encodes the chimeric MART-1 protein carrying the peptide signal and lysosomal sorting signal of human LAMP1 protein that probably target MART1 protein to MHC class II presentation pathway leading to a stimulation of CD4$^+$ T cells inducing a IFN-γ secretion. CD4$^+$ T-helper cells play also a key role in the establishment of the CTL response (33–35).
16. Only the intravenous route induces immunization with MART-1 mRNA Man-LPR. Surprisingly, the intramuscular and subcutaneous route did not induce protection against the tumor growth. Intraperitoneal injection gave significant protection but with a lower intensity than the intravenous one.
17. This vaccination schedule comprises 2 weekly injections of the vaccine before the tumor challenging. This does not imply that more injections could not give higher immunization.
18. The tumor volume (V) is calculated using $V = (L \times l)^2/2$, where l is the smallest diameter and L largest diameter of the tumor.
19. When L is equal to 410 mm.
20. Tumor-specific cytotoxic T lymphocytes (CTLs) are induced in lymphoid organs as spleen and afferent lymph nodes. Their presence in these organs evidences that a cellular immune response against tumor cells has been induced. After induction, CTLs migrate to the tumor site. MHC-I tumor antigen presentation by DCs in lymphoid organs allows the induction of CTLs that have the capacity to lyse tumor cells. The presence of CTLs is measured by the capacity of splenocytes to lyse tumor cells. For his purpose, the lactate dehydrogenase (LDH) colorimetric assay (CytoTox 96 Non-Radioactive

Cytotoxicity Assay, Promega) is used. It is an alternative to ^{51}Cr release cytotoxicity assays. LDH is a stable cytosolic enzyme that is released upon the lysis of tumor cells.

21. Cell lysis measurement is a sensitive assay. Spontaneous lysis that can occur during the cell preparation and cell culture can generate problems for the result interpretation. Thus it is important to introduce several control wells:
    (a) Wells containing tumor cells (target cells) without spenocytes (effector cells),
    (b) Wells containing various amount of spenocytes without tumor cells,
    (c) Wells with culture medium without any cells as the background control,
    (d) Wells that received Triton X-100 to lyse the totality of the cells in order to determine the maximum of LDH release.

    Moreover, data must be compared to the same experiments performed with splenocytes from nonimmunized mice.
22. Tumor-infiltrating CD8$^+$ and CD4$^+$ T cells both secrete IFN-γ. The presence of the induction of CTLs (CD8$^+$ T cells) against tumor cells is characterized by the secretion of IFN-γ by activated CD8$^+$ T-cells. The secretion of IFN-γ by CD4$^+$ T cells could be attributed to CD4$^+$CD25$^+$ regulatory T cells playing a crucial role in the suppression of the antitumor immune response.
23. Under these conditions, a few percent (3–8%) of CD8$^+$ IFN-γ positive cells as well as a few percent (3–8%) of CD4$^+$ IFN-γ positive cells are identified in CD8$^+$/ IFN-γ and CD4$^+$/ IFN-γ FITC versus PE dot plots, respectively.

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Chapter 17

Analysis of Survivin-Specific T Cells in Breast Cancer Patients Using Human DCs Engineered with Survivin mRNA

Özcan Met and Inge Marie Svane

Abstract

The observation that dendritic cells (DCs) charged with tumor-associated antigens (TAAs) is a potent strategy to elicit protective immunity in tumor-bearings hosts has prompted extensive testing of DCs as cellular adjuvant in cancer vaccines. To improve the clinical development of DC-based cancer vaccines, it may be beneficial to analyze preexistent immunity against TAAs in cancer patients because it may be easier to expand a memory pool of T cells compared to generating new immunity. Recent research shows that engineering DCs to synthesize tumor epitopes endogenously by transfecting DCs with mRNA-encoding TAAs are particular effective in stimulating robust T-responses in vitro and in vivo. In this chapter, we describe the methodology to analyze for survivin-specific T cells in breast cancer patients using human DCs engineered with survivin mRNA.

Key words: Dendritic cells, mRNA transfection, Cancer immunotherapy, Tumor-associated antigens, Vaccination

1. Introduction

Dendritic cells (DCs) are the chief orchestrators of innate and adaptive immunity (1). They process intracellular and extracellular proteins into antigenic peptides for subsequent presentation on cell surface major histocompatibility complex (MHC) molecules. Other cell types express MHC molecules and can be recognized by previously activated T cells, however unique attributes possessed by DCs enable them to trigger the expansion and differentiation of naive T cells into $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells (2). The important role of DCs in T-cell activation has prompted extensive testing of DCs as vehicles in attempts to manipulate the immune response for therapeutic effect, for example, in vaccination (3).

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Tumor antigens expressed either preferentially or exclusively by the tumor, collectively referred to as tumor-associated antigens (TAAs), can be recognized by the immune system (4). However, because tumors per se are not immunogenic, TAAs need to be channeled through DCs in order for the immune system to induce an effective T-cell response against the tumor (5). Thus, with the premise of converting the antigenicity of a growing number of tumors into immunogenicity, and translating this immunogenicity into tumor regression, the TAAs to be presented by DCs can be provided in the form of peptide, protein, tumor lysates, whole tumor cells, nucleic acid, and many more (3).

Comparative studies suggest that mRNA-based gene transfer is superior to other antigen-loading techniques in generating immunopotent DCs (6–9). With this approach, DCs can be manipulated to express intact TAAs, which are subjected to the full antigen processing and presentation cascade, in a manner indistinguishable from endogenous gene products. Furthermore, genetic modification of DCs is not limited to a single mRNA in fact, the full cohort of RNA expressed by a given tumor can be used to transfect DCs, for example, as a pool of in vitro amplified mRNA. The combination of unmatched safety and effective delivery to DCs renders mRNA transfection a promising genetic modality for cancer immunotherapy (10–13). While using cationic lipids to facilitate the uptake of mRNA by DCs, or mere incubation of DCs with naked mRNA, were reported to be sufficient for stimulation of antigen-specific T cells (14, 15), the application of electroporation as a means to introduce mRNA into DCs has been particularly efficient (6, 9, 16). High reproducibility and ease of performance in transfection of DCs are the inherent advantages of electroporation. Figure 1 depicts a representative experiment of DC electroporation with mRNA-encoding EGFP (see Note 1).

Information regarding the prevalence and magnitude of TAA-specific immune responses is important for identifying which TAAs are naturally targeted by the immune system and for understanding why natural immunity fails to eradicate tumors. Such TAAs could be targeted in DC-based treatment strategies because it may be easier to expand a memory pool of T cells compared to generating new immunity. TAAs that either play a role in the oncogenic process or promote cancer cell survival are favorable immunotherapeutic targets (17). Among the several dozen of such antigens, over-expressed anti-apoptotic proteins such as survivin are of particular interest as target antigen for the reason that downregulation or loss of the protein would severely inflict the growth potential of the cell and, thus, survivin is widely expressed in different tumor types, including breast cancer (18).

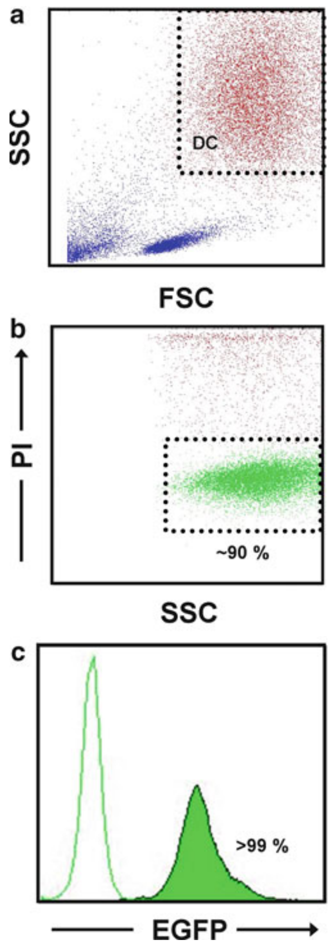

Fig. 1. Flow cytometry analysis of EGFP expression in DCs following electroporation with mRNA-encoding EGFP. Mature DCs were electroporated with 5 μg EGFP mRNA at 500 V, 2 ms and analyzed 24 h post-transfection. DCs are gated (*dotted square*) as cells exhibiting a large forward scatter and large side scatter profile (**a**) and cells are subsequently gated as propidium iodide negative (**b**) to exclude dead or dying cells. Expression of EGFP in viable DCs is shown in (**c**) (*filled histogram*) with mock-transfected (*empty histogram*) as control. The percentage of living DCs expressing EGFP is indicated.

Reconciling with this notion, spontaneous survivin-specific immune response has been identified as frequent features in many cancer patients and several survivin-based vaccination trials are currently ongoing at different institutes (18).

In this chapter, we describe the transfection of human DCs with survivin mRNA and its use for the analysis of survivin-specific T cells in primary breast cancer patients as depicted in Fig. 2.

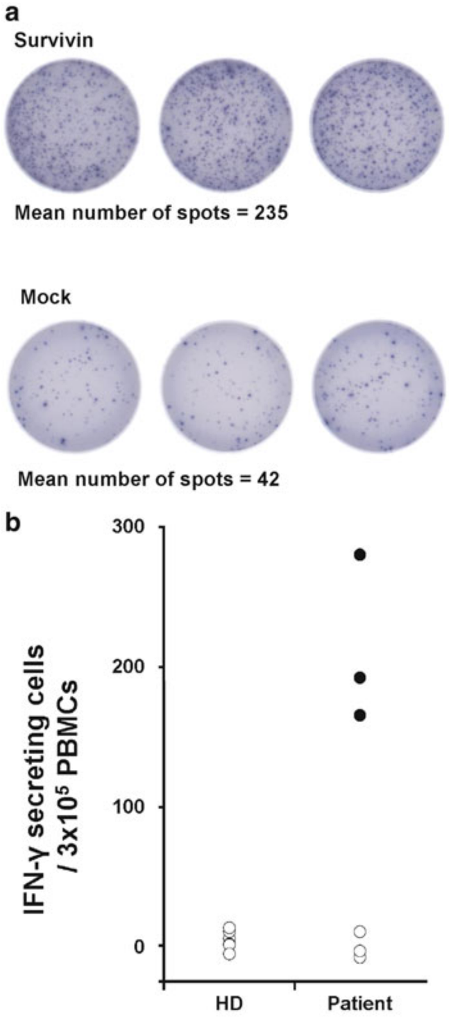

Fig. 2. Using survivin mRNA-transfected DCs to detect the frequency of survivin-reactive T cells in PBMCs from primary breast cancer patients. Example of an ELISPOT response against survivin in PBMCs from a primary breast cancer patient (**a**). Six patients and six healthy individuals were analyzed for survivin-specific T-cell reactivity (**b**). PBMCs from patients or from healthy individuals (HD) were stimulated with autologous DCs transfected with mRNA-encoding survivin for 7 days prior to being plated overnight with autologous mRNA-transfected DCs, which served as targets for survivin-specific T cells in the IFN-γ ELISPOT assay. Mock-transfected DCs were used as negative controls. A number of IFN-γ secreting cells were calculated using an automated ELISPOT reader. Each *circle* represents one individual. Responders (22) are marked as *filled circle*, whereas nonresponding individuals are marked as *open circle*. Data represents means of triplicates with mock-specific IFN-γ spots subtracted.

2. Materials

2.1. In Vitro Synthesis of Survivin mRNA

1. pSP73/survivin/A64 serves as template DNA vector for in vitro transcription (see Fig. 3, Note 2).
2. Qiagen Plasmid *plus* Maxi Kit (cat. nr. 12965, Qiagen, Hilden, Germany).
3. Restriction enzyme *Spe*I kit incl. BSA and NEB buffer 4 (cat. nr. R0133S, New England Biolabs, Ipswich MA, USA).
4. Nuclease-free water.
5. Heating block.
6. Wizard DNA Clean-Up System (cat. nr. A7280, Promega, Madison WI, USA).
7. mMESSAGE mMACHINE T7 Ultra Kit (cat. nr. AM1345, Ambion, Austin TX, USA).
8. MEGAclear Kit (cat. nr. AM1909, Ambion).
9. Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto CA, USA).
10. RNA 6000 Nano LabChip Kit (cat. nr. 5067-1511, Agilent Technologies).

2.2. Generation of Monocyte-Derived Human DCs

1. Heparin-coated blood collection tube.
2. Peripheral blood from breast cancer patient.
3. LymphoPrep™ solution.
4. PBS solution.

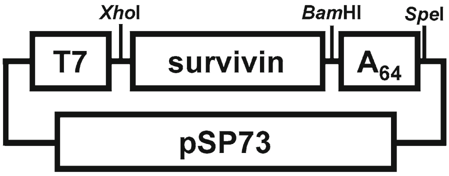

Fig. 3. The pSP73/survivin/A64 plasmid contains a 429-bp fragment encoding survivin followed by 64 A-T bp chain for addition of a poly(A) tail to the in vitro transcribed RNA. The transcription is controlled by a bacteriophage T7 promoter and at the 3′ end of the poly(A) tail a unique *SpeI* site is present that allows linearization of the plasmid DNA for in vitro transcription.

5. Refrigerated Sigma 6K10 centrifuge.
6. X-VIVO 15 medium (cat. nr. 04-418Q, Lonza, Basel, Switzerland).
7. 10 and 50 ml conical tubes.
8. Pasteur pipettes.
9. Hemocytometer.
10. Trypan blue.
11. 6-well cell culture plate.
12. Human AB-serum.
13. Humidified CO_2 incubator.
14. Recombinant human (rh) GM-CSF.
15. rh IL-4.
16. rh IL-1β.
17. rh TNF-α.
18. rh IL-6.
19. Prostaglandin PGE_2.
20. DMSO.
21. Mr. Frosty freezing container (cat. nr. 5100-0001, Nalgene, Thermo Fisher Scientific, Rochester NY, USA).
22. Isopropanol.
23. –80°C Freezer.

2.3. Electroporation of DCs

1. 24-well cell culture plate.
2. Opti-MEM medium (cat. nr. 11058-021, Invitrogen, Paisley, UK).
3. 2 ml Eppendorf tubes.
4. BTX ECM 830 square-wave electroporator (Harvard Apparatus, Holliston MA, USA).
5. 4-mm gap cuvettes.
6. Autologous mature DCs generated from breast cancer patient.
7. 10 ml conical tubes.
8. X-VIVO 15 medium (Lonza).
9. Hemocytometer.
10. Trypan blue.
11. Refrigerated Sigma 6K10 centrifuge.
12. Humidified CO_2 incubator.

2.4. Stimulation of Survivin-Specific T Cells

1. 24-well cell culture plate.
2. 37°C water bath.
3. Cryovials with autologous PBMCs from breast cancer patient.
4. rh IL-2.
5. 10 and 50 ml conical tubes.
6. X-VIVO 15 medium (Lonza).
7. Hemocytometer.
8. Trypan blue.
9. Refrigerated Sigma 6K10 centrifuge.
10. Humidified CO_2 incubator.

2.5. Analysis of Survivin-Specific T Cells

1. Multiscreen 0.45 μm Filter plates for ELISPOT (cat. nr. MSIPN4W50, Millipore, Billerica MA, USA).
2. Multichannel pipette.
3. Human Cytomegalovirus, Epstein Barr virus and Influenza virus (CEF) peptide pool (cat. nr. 3615-1, Mabtech, Nacka Strand, Sweden).
4. Anti-human IFN-γ mAb (1-D1K) (cat. nr. 3420-3-1000, Mabtech).
5. Anti-human IFN-γ mAb-biotinylated (7-B6-1) (cat. nr. 3420-6-1000, Mabtech).
6. Streptavidin-ALP (Mabtech).
7. BCIP/NBT^{plus} substrate solution (cat. nr. 3650-10, Mabtech).
8. Washing buffer: PBS, 0.05% Tween-20.
9. Diluting buffer: PBS, 1% BSA, 0.02% NaN_3.
10. Distilled water.
11. ImmunoSpot 2.0 Analyzer (CTL, Cleveland OH, USA).
12. Opti-MEM medium (Invitrogen).
13. 2 ml Eppendorf tubes.
14. In vitro transcribed survivin mRNA.
15. BTX ECM 830 square-wave electroporator (Harvard Apparatus).
16. 4-mm gap cuvettes.
17. 10 ml conical tubes (Nunc).
18. X-VIVO 15 medium (Lonza).
19. Hemocytometer.
20. Trypan blue.

21. Refrigerated Sigma 6K10 centrifuge.
22. Humidified CO_2 incubator.

3. Methods

3.1. In Vitro Synthesis of Survivin mRNA

1. Purify the pSP73/survivin/A64 plasmid (see Note 2) with the Qiagen Plasmid *plus* Maxi Kit according to the manufacturer's protocol (see Note 3).
2. For linearization of the pSP73/survivin/A64 plasmid prepare the following in one Eppendorf tube:
   (a) 29.5 µl Nuclease-free water (pre-warmed to 37°C)
   (b) 5.0 µl NEB buffer 4 (10×) (pre-warmed to 37°C)
   (c) 5.0 µl BSA (10×) (pre-warmed to 37°C)
   (d) 10.0 µl pSP73/survivin/A64 plasmid (2 µg/µl)
   (e) 0.5 µl *Spe*I restriction enzyme (10 U/µl)
3. Incubate for 1 h in a heating block at 37°C.
4. Purify linearized pSP73/survivin/A64 plasmid with Wizard DNA Clean-Up System according to the manufacturer's protocol.
5. Produce survivin mRNA with mMessage mMACHINE Ultra T7 Ultra Kit according to the manufacturer's protocol (see Note 4).
6. Purify survivin mRNA transcripts with the MEGAclear Kit according to the manufacturer's protocol.
7. Evaluate the mRNA length, concentration and purity with the Agilent 2100 Bioanalyzer and the RNA 6000 Nano LabChip Kit according to the manufacturer's protocol (see Note 5).

3.2. Generation of Monocyte-Derived Human DCs

Because peripheral blood mononuclear cells (PBMCs) are easily collected through leukapheresis or via buffy coat preparations, one of the best-characterized protocols for generating mature human DCs involves differentiation of DCs from PBMCs. In this protocol, monocytes which are enriched by plastic adherence, elutriation or $CD14^+$ immunomagnetic selection are cultured for 5–7 days to generate immature DCs (19) , followed by 1–2 days of culture in the presence of IL-1β, IL-6, TNF-α, and PGE_2 to induce their maturation (20). Upon maturation, DCs express high levels of MHC–peptide complexes and costimulatory molecules, stimulate potent T-cell responses, and possess migratory function in vitro.

1. After informed consent, take 60 ml blood from a breast cancer patient at the time point of primary diagnosis in heparin-coated blood collection tube.

2. All procedures are subsequently performed under sterile conditions in a flow cabinet and with sterile reagents.
3. Dilute the blood sample 1:1 with PBS in 50 ml conical tubes.
4. Next, layer the blood carefully onto lymphoprep using a 10 ml plastic pipette, typically 30 ml of diluted blood over 20 ml of lymphoprep in 50 ml conical tubes, and promptly centrifuge at 1,200 × *g* for 25 min at room temperature without acceleration and break to prevent disruption of the gradient.
5. Following centrifugation, a layer of PBMCs is found between the interface of the lymphoprep and blood plasma. Carefully remove the cells with a Pasteur pipette, making sure not to disturb the gradient and place the cells into new 50 ml conical tubes.
6. Add PBS until a final volume of 50 ml.
7. Switch acceleration and brake on and centrifuge for 5 min at 400 × *g* to washout lymphoprep (see Note 6).
8. Discard supernatant, resuspend the cell pellet, and add PBS until a volume of 50 ml.
9. Repeat step 8.
10. Discard supernatant, resuspend in X-VIVO 15 medium and pool the cells in one 50 ml tube.
11. Count the cells with a hemocytometer, using Trypan blue stain (mixed with the cell sample at a 1:1 ratio) to discriminate between live and dead cells. Dead cells appear blue under light microscope as the cell membranes are permeable to the blue dye.
12. Adjust PBMCs to 3×10^6 cells/ml in X-VIVO 15 medium and culture cells in 6-well plates for 1.5 h at 37°C in humid 5% CO_2 incubator. A volume of 3 ml of PBMCs/well is added to two wells (see Note 7).
13. Place the tube with the remaining PBMCs on ice (they will be cryopreserved for later use).
14. After 1.5 h, harvest the medium with non-adherent cells without disrupting the adherent cells, by gently swirling the 6-well plate for 30 s and transfer non-adherent cells to the tube with the remaining PBMCs on ice. The non-adherent cell population will now mainly consist of peripheral blood lymphocytes (PBLs).
15. Wash the wells by adding 3 ml X-VIVO 15 medium on the side of the well and gently swirl the 6-well plate for 30 s. Discard the medium.
16. Add 3 ml X-VIVO 15 medium supplemented with 2% AB-serum (heat inactivated, see Note 8), 250 U/ml rh IL-4 and 1,000 U/ml rh GM-CSF, and incubate for 6 days at 37°C in 5% CO_2 to generate immature DC (see Note 9).

17. Centrifuge the tube with the remaining PBMCs including PBLs for 5 min at 400 × *g* and put on ice.
18. Prepare freezing medium consisting of 90% AB-serum and 10% DMSO. Make 1 ml freezing medium for every 1×10^7 PBMCs counted in step 11. Put on ice for 5 min (see Note 10).
19. Resuspend cell pellet with freezing medium and transfer 1 ml of 1×10^7 PBMCs into a designated cryovial and promptly transfer the cryovial into a pre-cooled Mr. Frosty-style freezing container that has been filled with 70% isopropanol according to the manufacturer's instructions. Place the freezing container at –80°C freezer.
20. On day 6, add 1,000 U/ml rh IL-1β, 1,000 U/ml rh TNF-α, 1,000 U/ml rh IL-6, and 1 μg/ml PGE_2 to each well and allow DCs to mature for 24 h.

3.3. Electroporation of DCs

The electroporation method relies on the use of a transmembrane electric field pulse to induce microscopic pores (electropores) in the cell membrane (21). The presence of electropores allows molecules, e.g. mRNA, to pass from one side of the membrane to the other. If the electric field pulse has the proper parameters, the electropores reseal spontaneously and the electroporated cells will fully recover. Thus, for the transient expression of TAAs in DCs, electroporation is a straightforward, nontoxic, noninfectious and highly efficient gene delivery system.

1. Prior to electroporation, prepare a 24-well plate by adding 2 ml X-VIVO 15 medium in 6 wells; 3 wells for electroporation with mRNA and 3 wells for mock electroporation (i.e., without mRNA, see Note 11). Place the plate in 5% CO_2 at 37°C.
2. Harvest day 7 mature DCs by thorough pipetting and transfer cells into a 10 ml tube.
3. Add OPTI-MEM medium to final volume of 10 ml and centrifuge for 5 min at 400 × *g*.
4. Discard supernatant and wash the cells with 10 ml OPTI-MEM medium.
5. Discard supernatant and resuspend cells in OPTI-MEM medium.
6. Count the cells and adjust to 5×10^6 cells/ml.
7. Transfer 250 μl of the cell suspension into 2 sterile Eppendorf tubes and place the tubes on ice for 5 min (see Note 12).
8. During the incubation, set up the electroporation parameters (see Note 13):
   (a) Volt: 500
   (b) Pulse length: 2 ms

   (c) Pulse: 01
   (d) Interval: 01.0 s
   (e) Polarity: Unipolar
9. In one Eppendorf tube, mix 5 μg mRNA-encoding survivin and transfer cell suspension immediately into a 4-mm electroporation cuvette (see Note 14).
10. Insert the cuvette into the electroporation champer and trigger the pulse. Inspect that the electroporation is successful (see Note 15).
11. Transfer DCs immediately after electroporation into the prepared 24-well plate by adding 60 μl cell suspension into each designated well without excessive pipetting (see Note 16).
12. Repeat steps 9–11 with the second Eppendorf tube, but without mRNA.

3.4. Stimulation of Survivin-Specific T Cells

1. On DC culture day 7, place 25 ml X-VIVO 15 medium in 50 ml polypropylene tube in a 37°C water bath for about 30 min.
2. Transfer 2 cryovials of 1×10^7 PBMCs from −80°C freezer and place directly on 37°C water bath (see Note 17).
3. Hold cryovials in the surface of the water bath until cell suspension is almost completely melted or a small bit of ice remains.
4. Dry off the outside of the cryovials and wipe with 70% ethanol.
5. Add 1 ml of 37°C warm X-VIVO 15 medium to the thawed cells dropwise.
6. Transfer the cell suspension to the 50 ml polypropylene tube containing 25 ml of warm media.
7. Centrifuge the cells for 5 min at $400 \times g$.
8. Discard supernatant, resuspend in 10 ml X-VIVO 15 medium.
9. Check for clumps and remove them with a pipettor tip. Repeat step 7.
10. Repeat steps 11–12 in Subheading 3.2.
11. After 1.5 h, harvest the non-adherent PBLs and adjust the cells to 3×10^7 cells/ml.
12. Repeat steps 15, 16, and 20 in Subheading 3.2 to generate another batch of DC, which will be used for restimulation of the non-adherent PBLs in Subheading 3.5 (see Note 18).
13. Add 100 μl of PBLs pr. well to the 24-well plate with mRNA-transfected DCs (this will result in an effector:target ratio of 10:1 (i.e., 3×10^6 PBLs:3×10^5 DCs)) and incubate at 37°C in 5% CO_2.
14. Next day, add 40 U/ml IL-2 to each well and incubate for additional 6 days at 37°C in 5% CO_2.

3.5. Analysis of Survivin-Specific T Cells

1. Dilute the coating antibody (anti-IFN-γ) to 7.5 μg/ml in sterile PBS.
2. Add 75 μl of diluted antibody to 3×4 wells of a 96-well Multiscreen ELISPOT plate for detection of IFN-γ release of PBLs stimulated with (a) survivin mRNA-transfected DCs, (b) mock-transfected DCs (negative control of experiment,) and (c) CEF peptide pool (positive control of ELISPOT) (see Note 19). Make sure that all wells are covered. Incubate overnight at room temperature.
3. Next day, decant primary antibody solution and wash the plate six times with 200 μl PBS (see Note 20).
4. Add 200 μl of blocking solution (X-VIVO 15 medium) to each designated well to saturate remaining binding sites. Incubate for 2 h at room temperature.
5. During blocking of the ELISPOT plate, harvest PBLs after 7 days of coculture with mRNA-transfected DCs by gentle pipetting and transfer cells into a 10 ml tube (see Note 21).
6. Add X-VIVO 15 medium to a final volume of 10 ml and centrifuge for 5 min at 400×*g*.
7. Discard supernatant and resuspend cells in X-VIVO 15 medium.
8. Count the cells and adjust to 3×10^6 cells/ml.
9. Discard the blocking solution and add 100 μl of cell suspension to each of the designated wells.
10. Place the ELISPOT plate at 37°C in 5% CO_2 and incubate for 30 min.
11. During the incubation, harvest the second batch of mature DCs generated in Subheading 3.4.
12. Prepare two 10 ml tubes with 3,100 μl of 37°C warm X-VIVO 15 medium. One is for survivin mRNA transfection, the other is for mock-transfection.
13. Electroporate DCs with mRNA by repeating steps 2–10 and 12 in Subheading 3.3.
14. Transfer DCs immediately after electroporation into the 10 ml tube with prevarmed X-VIVO 15 medium and incubate for 15 min.
15. Add 100 μl of mRNA-transfected DCs to the designated wells in the ELISPOT plate.
16. Dilute the CEF-peptide pool to 10 μg/ml in X-VIVO 15 medium.
17. Add 100 μl of CEF-peptide to the designated wells in the ELISPOT plate.
18. Incubate the cells for 18 h at 37°C in 5% CO_2. Do not move the plates while the cells are culturing. This will lead to spots that will not be well defined.

19. Remove the cells and unbound cytokine by emptying the plate and wash six times with 200 μl PBS 0.1% Tween 20. Shake excess fluid and pat dry.
20. Dilute the biotinylated secondary antibody (7-B6-1) to 1 μg/ml in diluting buffer (PBS, 1% BSA, 0.02% NaN_3). Add 75 μl/well and incubate for 2 h at room temperature.
21. Empty the plate and wash six times with 200 μl PBS 0.1% Tween 20. Shake excess fluid and pat dry.
22. Dilute the streptavidin–ALP (1:1,000) in diluting buffer and add 75 μl/well. Incubate for 1 h at room temperature.
23. Empty the plate and wash six times with 200 μl PBS 0.1% Tween 20 followed by one wash in PBS. Shake excess fluid and pat dry.
24. Add 75 μl of substrate solution (BCIP/NBT) and incubate for 5–15 min at room temperature until blue spots develop (see Note 22).
25. Stop color development by washing extensively in distilled water.
26. Place the ELISPOT plate with the membrane upwards on a paper towel and leave the plate to dry overnight in the dark.
27. When plate is completely dry, inspect and count spots in an ELISPOT reader such as the ImmunoSpot 2.0 Analyzer.
28. Store plate in the dark at room temperature.

4. Notes

1. Because of its sensitivity and relative simplicity in detection by flow cytometry, EGFP has often been used as a model antigen in establishing an effective electroporation procedure for gene transfer of DCs. Several groups, including ours, have demonstrated that EGFP is remarkable stable when introduced genetically into DCs and were readily detected 3–5 days post-electroporation (9, 16). However, this stability in antigen expression is probably unlike most TAAs targeted in vaccination strategies, which in general have a high protein turnover (22, 23).
2. The plasmid pSP73/survivin/A64 has been made as follows: the coding sequence of survivin was excised from the pGEM-sig/survivin/DClamp plasmid using *Bam*HI, and a *Xho*I site is added at 5′ end resulting in a survivin fragment flanked by *Xho*I spanning the start codon at the 5′ end and a *Bam*HI site at 3′ end. This fragment was then cloned into the vector pSP73/A64 (24) using *Xho*I and *Bam*HI restriction sites to

generate pSP73/survivin/A64 (Fig. 3). The pSP73/survivin/A64 plasmid was subsequently propagated in *Escherichia coli*.

The plasmid pGEM-sig/survivin/DClamp was kindly by provided by Professor K. Thielemans, Medical School of the Vrije Universiteit Brussel.

Vector pSP73/A64 was kindly provided by Professor E. Gilboa, Duke University Medical Center.

3. A yield of 400–500 μg purified pSP73/survivin/A64 plasmid is usually obtained at this step, which is more than enough for the downstream applications, i.e., linearization, in vitro transcription and transfection.
4. A typical yield of 20–25 μg ARCA-capped survivin mRNA transcripts is obtained at this step.
5. This system provides a more precise description of the integrity and quantity of the mRNA sample compared to the traditionally used agarose gel electrophoresis and spectrophotometry. Figure 4 depicts the result of a representative electrophoretic chip run. The electropherogram and gel-like image provide data on size and concentration of mRNA, and also show the presence of degradation products in the mRNA sample. Only highly pure mRNA preparations should be used for electroporation of DCs.
6. High speed is required to prevent cell loss, since the remaining lymphoprep increases the density of the solution.
7. In this protocol, monocytes are isolated from PBMCs by exploiting their ability to adhere to plastic. This is a quick, cheap, and easy procedure. In addition, plastic adherence can induce cell activation which might be beneficial in some systems. However, contaminating lymphocytes are often present in the culture.
8. The purpose of heat inactivation is to eliminate protease and complement proteins which can be interfering substances when it is used in cell culture systems. However, a careful controlled process must be followed in order to avoid damaging the cell growth promoting properties of the serum and reducing the formation of unwanted precipitates. Bring the temperature of the serum to 56°C in a shaking water bath and hold it there for 30 min. The shaking will help avoid the formation of protein and other forms of precipitates. Then cool the serum to room temperature prior to use. Some precipitation may be evident, which can be removed by filtration.
9. Some investigators add autologous human AB-serum (2–10%) during the adherence step, for the reason that cells adhere harder in the presence of serum. In our experience, however, monocytes adhere more quickly in the absence of serum.

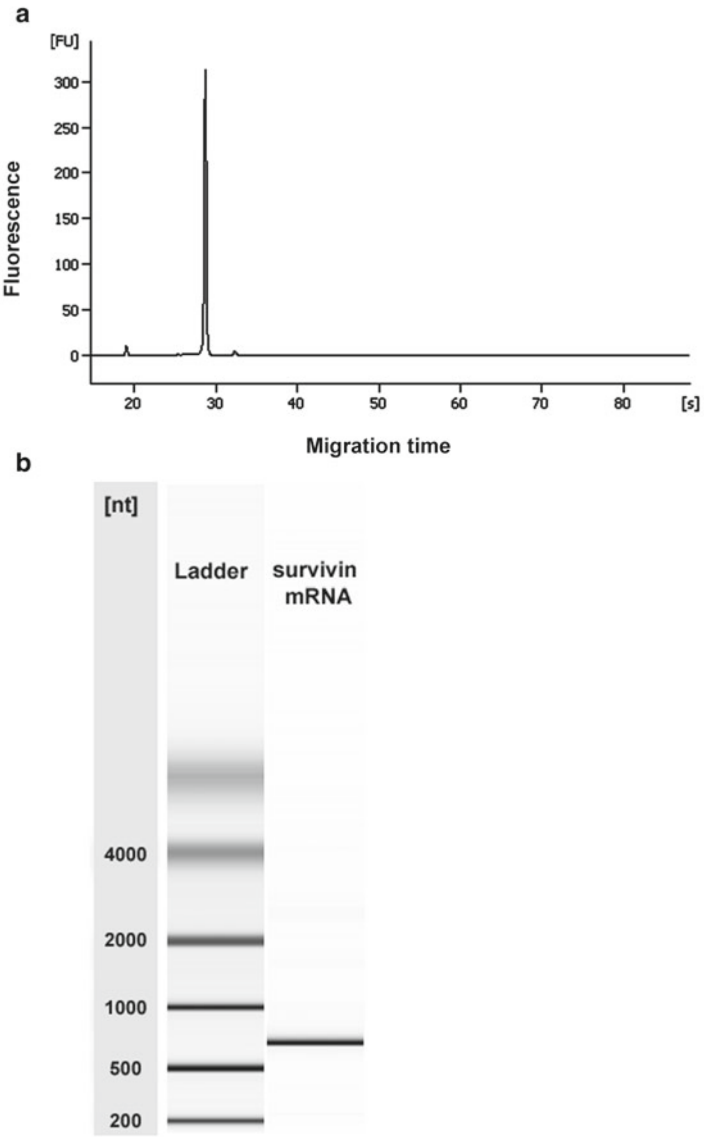

Fig. 4. Quality control of survivin mRNA transcript. The quality of the in vitro transcribed mRNA was analyzed using the Agilent 2100 Bioanalyzer. An electropherogram (**a**) and a gel-like image of survivin mRNA is used to calculate the mRNA concentration, to check for any mRNA impurities and to analyze the length of the survivin mRNA.

10. Cryoprotectants, such as DMSO, reduce the amount of ice present during freezing and reduce solute concentration, thereby reducing ionic stress. However, these compounds can also cause osmotic injury since they are hypertonic and can cause damage during their addition or removal.

11. Since many pathogen recognition receptors within DCs can detect RNA and thus trigger its activation, it may be more proper to use mRNA encoding an irrelevant antigen (such as EGFP) as a negative control. However, in our hands transfection of DCs with or without mRNA does not affect the maturation state of DCs or the expression pattern of cytokines produced by transfected DCs in an antigen-unspecific manner. What we have observed is that manipulating the electroporation conditions can have an impact on certain surface markers on DCs as well as transgene expression, which leads to a more potent T-cell stimulation (9).
12. It is important to incubate cells on ice prior to electroporation as the pulse might cause thermal shock to the cells and thus negatively impact cell survival.
13. Both exponential decay and square wave pulses can be used to transfect DCs. However, while exponential decay pulses are more ideal to transfect bacteria and yeast, the square wave pulses are better accepted by more delicate mammalian cells such as DCs. With the square wave pulse there is a period of homeostasis to be reached in the cells before the wave is removed. As a result, there is a lower mortality rate in cells while maintaining transfection efficiencies. In our hands, transfecting using exponential decay pulses resulted in much higher rate of cell mortality in DCs than using square wave pulses.
14. Add mRNA to the cell suspension just before the electroporation pulse to avoid degradation due to possible RNAses. Also make sure that no air bubbles are present in the suspension before or during delivery into the cuvette as this can cause arcing.
15. Immediately after the pulse, small air bubbles or a small layer of foam should be apparent at the top of the cell suspension. This signals that the current has passed through the solution and that the electroporation is successful.
16. After electroporation cells should be transferred immediately to 37°C warm cell culture medium. Below 37°C, the resealing of the membranes is delayed after electroporation, so that over a longer period of time an exchange of molecules takes place between the cell and its environment. This causes increased stress and can reduce the survival rate especially when electrolysis occur. Also avoid repetitive pipetting as cells are very fragile after electroporation.
17. It is important for cell viability that the cells are thawed and processed quickly; thawing should take less than a minute. If PBMCs are not thawed properly, viability and cell recovery can be compromised and cells may not perform optimally in functional assays. In general, cells should be thawed quickly but

diluted slowly to remove DMSO. Cells with DMSO intercalated into their membranes are very fragile and must be pelleted and handled gently.

18. We recommend to thaw two ampules of PBMCs and then to use the adherent portion of the cells to generate DCs and the non-adherent portion (PBLs) as responder cells.

    The DCs are subsequently transfected with survivin mRNA and used in the ELISPOT assay for restimulation of the PBLs in Subheading 3.5. This will increase the sensitivity of the ELISPOT assay.

19. Because cryopreservation of cells might affect their functional state, it is important to check that the effector cells to be analyzed are indeed functional. The CEF peptide pool contains 23 different class-I restricted peptides spanning a wide range of HLA-types—all defined as common $CD8^+$ T-cell epitopes derived from cytomegalovirus, Epstein-Barr virus and influenza virus. It efficiently induces IFN-γ production by virus-specific $CD8^+$ T cells in almost 90% of Caucasians and thus functions as a useful control to evaluate responsiveness of T cells in the ELISPOT assay.

20. Washing is easily accomplished using a multichannel pipette and by dunking the plate at an angle in the wash solution, shaking excess liquid from the plate, and patting the bottom dry on absorbent paper (i.e., towel paper) after the final wash.

21. One can purify $CD4^+$ and $CD8^+$ T lymphocytes by $CD4^+$ and $CD8^+$ T cell isolation kit prior to the second stimulation with survivin-transfected DCs to address which T-cell subpopulation are responsible for the secretion of IFN-γ in the ELISPOT assay.

22. For optimal results filter the substrate solution (0.2 μm) before use as precipitation of the substrate does occur over time.

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Chapter 18

mRNA Electroporation as a Tool for Immunomonitoring

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Abstract

Monitoring the immune response is an essential aspect of numerous clinical vaccination trials in order to evaluate the efficacy. In these clinical vaccination trials, peripheral blood mononuclear cells (PBMC) are isolated at different time points from patient blood samples and subsequently cryopreserved to allow batch analysis at a later time point. Here, we present a newly developed short-time assay which allows direct ex vivo analysis of multi-epitope antigen-specific immune responses using mRNA electroporation of cryopreserved PBMC. This novel method is a rapid and elegant tool and will be convenient for monitoring the cellular immune status of patients in clinical vaccination settings.

Key words: mRNA, Electroporation, PBMC, Cryopreservation, Immunomonitoring

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1. Introduction

To date, the number of immunotherapeutic strategies in clinical trials increases exponentially. In such therapeutic vaccination trials, the capability of the vaccine to induce specific immune responses needs to be monitored before and after vaccination in order to assess the potency of the vaccine with an immunological test correlating with the clinical outcome. In this respect, we fervently need cellular immunomonitoring tools. Several methods have been developed to evaluate T cell-mediated immune responses in clinical vaccination trials, such as Elispot and intracellular cytokine flow cytometry (1–5). With specific regard to immune-monitoring assays in multicenter vaccination trials, peripheral blood is drawn at specific time points before, during, and after the vaccine cycle. Subsequently, isolated PBMC are

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cryopreserved to allow batch analysis at a later time point. With regard to immune-monitoring assays it is of utmost importance that PBMC maintain their capability to recall immune responses after cryopreservation (6, 7).

Here, we present a newly developed short-time assay which allows direct ex vivo analysis of multi-epitope antigen-specific T cell immune responses. This novel method is based on mRNA electroporation of isolated, cryopreserved, and uncultured peripheral blood mononuclear cells (PBMC). In doing so, we have demonstrated that thawed mRNA-transfected PBMC are sufficiently potent to allow direct analysis of antigen-specific T cell responses present in blood, in a short-time assay and without the need for prolonged in vitro stimulation or T cell enrichment (8).

All reported methods require in vitro stimulation using autologous antigen-presenting cells, such as dendritic cells (DC), prepared from PBMC to evaluate T cell-mediated immune responses. In this perspective, current method provides several advantages because it obviates the need for in vitro cultured antigen-presenting cells and because an overnight incubation is sufficient for activation of T cells. Indeed, electroporation of PBMC compared to the conventional mixed lymphocyte peptide cultures described by Greiner et al. (9) and Li et al. (10) is a much more rapid and less labor-intensive alternative for detection of recall immune responses. In addition, aside from stimulation of recall responses, which is preferred in an immunomonitoring setting, DC also initiate primary responses (11), which might result in overestimation of the response detected in vitro. Moreover, while others (12, 13) have shown that mRNA-transfected PBMC can be used as alternative APC source to induce antigen-specific cytotoxic T cells in vitro, we show that an overnight incubation of the electroporated PBMC is enough to activate (memory) T cells.

A variety of stimulatory antigen sources has been used, including proteins (14), single synthetic peptides (15) or overlapping peptide pools (16), viral lysates (17), DNA or viral vectors (18). Previously, we have demonstrated that transfection of mRNA by electroporation is a safe, highly efficient and clinically applicable method for antigen expression (19–21). Moreover, it was recently demonstrated that mRNA could be efficiently electroporated into PBMC (22, 23) and that mRNA-transfected PBMC were able to induce antigen-specific cytotoxic T lymphocytes in vitro (12, 13). Furthermore, the use of (tumor) antigen-encoding mRNA will broaden the immune response because it covers every epitope with potential relevance. Indeed, the use of mRNA coding for the full antigenic spectrum of a protein allows coverage of every epitope with potential relevance instead of a single epitope covered by HLA-restricted peptides or peptide pools. As demonstrated by Yao et al. (24), antigen-specific T cell frequencies vary according to T cell epitopes. This can lead to an underestimation of the magnitude and diversity of the T cell response.

2. Materials

2.1. Solutions/Saline Solutions

1. Phosphate-buffered saline (PBS), pH 7.4, no Ca^{2+}, no Mg^{2+} supplemented with 1 mM EDTA.
2. Heat-inactivated FBS: 30 min at 56°C.
3. Heat-inactivated human AB serum: 30 min at 56°C.
4. OptiMEM w/o phenol red pH indicator.

2.2. Gradients

Ficoll density gradient solution: density 1.077 ± 0.001 g/mL.

2.3. Freezing Medium

Heat-inactivated fetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO). Sterile filter through a 0.2 μm filter. Freezing medium can be made up in advance, aliquoted in sterile 50 mL tubes and stored at 4°C for up to 2 weeks.

2.4. Collection Medium

Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS and 50U/mL DNase.

2.5. Culture Medium

IMDM supplemented with L-glutamine (2 mM), HEPES (25 mM), gentamicin (10 mg/L), amphotericin B (1 mg/L), and 5% human AB serum.

2.6. Equipment

1. Cryo 1°C freezing container filled with isopropyl alcohol.
2. Tabletop centrifuge consisting of a rotor and buckets capable of holding 50 mL tubes and reaching 740 × *g*.
3. Warm water bath.
4. 5% CO_2 humidified incubator.
5. Spectrophotometer.
6. Automated spot-counting system.
7. (Automated) Hemocytometer.
8. Electroporation device.

2.7. Other

1. 50 mL Conical tubes.
2. 4 mm Electroporation cuvettes.
3. 96-Well PVDF plates.
4. 5′ Capped mRNA.

3. Methods

3.1. Isolation of Peripheral Blood Mononuclear Cells from Human Blood (see Note 1)

1. Pipette heparinized blood from the vacutainers and pool into a larger sterile container (e.g., tissue culture flasks).
2. Rinse each vacutainer with Ca^{2+}/Mg^{2+}-free PBS, thereby diluting the blood at a 1:1 ratio (e.g., add 10 mL PBS to 10 mL blood), and add it to the pool. Gently mix the blood with the PBS.
3. In sterile 50 mL conical tubes, use approximately 15 mL ficoll for 25–30 mL diluted blood. Ficoll may be slowly underlayed beneath blood, using a 10 mL pipette and taking care not to mix the two layers; or blood may be overlayed above the ficoll, again using caution and proceeding slowly, so as not to mix the two layers.
4. Centrifuge the samples for 30 min at $740 \times g$ at room temperature, with the brake OFF so that the deceleration does not disrupt the density gradient.
5. After centrifugation, using a sterile pipette, collect the white cells at the interface between the ficoll below and the plasma above, this should be around 10 mL. Do not collect too much ficoll as this will decrease cell yield after the washes. Avoid also the red blood cell pellet at the bottom of the tube. Transfer white blood cells from each ficoll tube into a new, sterile 50 mL tube.
6. Dilute PBMC with PBS to wash cells. Use AT LEAST double volume PBS to cells for the wash.
7. Centrifuge cells at $330 \times g$ for 10 min at room temperature to pellet. This time the brake of the centrifuge should be ON.
8. Decant and discard supernatant and collect cell pellet by resuspending cells in PBS (see Note 2). Cells can be pooled into one tube.
9. Count an aliquot of the cells to be frozen using a (automated) hemocytometer and calculate the total number of PBMC collected (PBMC/mL × total volume of cells).
10. Centrifuge cells at $330 \times g$ for 10 min at room temperature to pellet.

3.2 Freeze/Thaw Procedure of Peripheral Blood Mononuclear Cells

1. Resuspend the pelleted cells to the appropriate concentration in the chilled freezing medium consisting of FBS supplemented with 10% DMSO (see Note 3). Aliquote approximately 10×10^6 PBMC per mL per cryovial.
2. Transfer the cryovials containing the cells to the cryo-freezing container filled with isopropyl alcohol (see Note 4), and place

immediately in the −80°C freezer. After 24 h, cryovials should be transferred to a liquid nitrogen freezer for indefinite storage (see Note 5).

3. In order to thaw PBMC, prepare warm 25 mL of collection medium (i.e., IMDM supplemented with 10% FBS and 50 U/mL DNase (see Note 6)). For this, heat the collection medium in a warm water bath to 22–37°C before adding to the cells.
4. Thaw cryovials directly from freezer into a warm water bath (see Notes 7 and 8).
5. Transfer the content of the cryovial to a 50 mL conical tube and rinse the vial with 1 mL warm collection medium. Slowly, drop-by-drop, add the rinse to the cells in the 50 mL tube. Subsequently, add slowly (over 30 s to 1 min) 8 mL of warm collection medium to the 50 mL tube (see Note 9). If desired, different cryovials of one donor can be pooled in one 50 mL tube.
6. Centrifuge cells at 330 × *g* for 10 min at room temperature to pellet with the brake of the centrifuge ON.
7. Decant supernatant, and carefully resuspend cell pellet in 10 mL warm collection medium per cryovial thawed.
8. Count the cells and keep them at 37°C in a 5% CO_2 humidified incubator for 12–18 h (see Note 10).

3.3. mRNA Electroporation of Peripheral Blood Mononuclear Cells

1. Put the power of the electroporation device ON and install the following settings: square-wave pulse, voltage = 500 V, time = 5 ms, and cuvette = 4 mm (see Note 11).
2. Prepare culture medium (i.e., IMDM supplemented with 5% human AB serum) for collection of cells after electroporation and keep at room temperature.
3. Collect the thawed and overnight rested PBMC.
4. Centrifuge cells at 330 × *g* for 10 min at room temperature to pellet with the brake of the centrifuge ON.
5. Decant supernatant, and carefully resuspend cell pellet in 5 mL OptiMEM medium per initial cryovial thawed.
6. Count an aliquot of the cells to be electroporated using a (automated) hemocytometer and calculate the total number of PBMC collected (PBMC/mL × total volume of cells).
7. Centrifuge cells at 330 × *g* for 10 min at room temperature to pellet with the brake of the centrifuge ON.
8. Decant supernatant, and resuspend cell pellet at 10×10^6 PBMC per 100 μL OptiMEM (see Note 12).
9. Determine the number of electroporations (see Notes 13 and 14) and use 200 μL of cell suspension per electroporation (see Note 15).

10. Transfer the cell suspension (min. 200 µL and max. 500 µL) in a 4 mm electroporation cuvette.
11. Thaw the required amount of mRNA (see Notes 16 and 17) and add to the electroporation cuvette.
12. Place the cuvette in the electroporation device and push the appropriate button to electroporate.
13. Immediately after electroporation add a small amount of culture medium to the cuvette using a Pasteur pipette and transfer cells to a 50 mL conical tube containing 10 mL of culture medium.
14. Count the cells and keep them at 37°C in a 5% CO_2 humidified incubator for maximum 2 h until further use.

3.4 Detection of Cytokine-Producing Antigen-Specific T Cells Using Elispot

1. Use anti-cytokine pre-coated PVDF plates as supplied in a kit or coat a non-pre-coated PVDF plate according to the supplier's suggested guidelines 1 day in advance to current procedure (see Notes 18 and 19).
2. Collect the cells.
3. Centrifuge cells at 330 × *g* for 10 min at room temperature to pellet with the brake of the centrifuge ON.
4. Decant supernatant, and resuspend cells at 2.5×10^5 to 5×10^5 PBMC/100 µL culture medium (see Note 20).
5. Slowly pipet 100 µL of cell suspension into the wells of the anti-cytokine-coated plates, and avoid air bubbles.
6. Incubate the cells undisturbed at 37°C in a 5% CO_2 humidified incubator for 23–36 h (see Note 21).
7. For the development of the Elispot plates (i.e., making the cytokine-producing cells visible as colored spots) follow the suggested guidelines from the supplier of the kit used.
8. Dry the plate (see Note 22).
9. The plate is ready to be analyzed on an automated spot counter or can be stored for later analysis at room temperature in the dark (see Note 23).

4. Notes

1. PBS and ficoll must be sterile and at *room temperature* for use in PBMC separation step.
2. The expected cell count for healthy human donors is 1–2 million PBMC for each mL of blood drawn.

3. Cryoprotective reagents, such as DMSO, reduce the formation of ice crystals during the freezing procedure as well as ionic stress. However, since these components are hypertonic they can cause osmotic damage when supplemented or removed. Therefore, it is necessary to freeze down cells rapidly, while diluting the cells slowly in order to remove DMSO. Cells containing DMSO in their membrane are very fragile and should be processed with extreme caution.
4. For cryo-freezing containers, follow the suggested guidelines and fill containers to fill line with isopropyl alcohol.
5. Extended storage at −80°C will eventually result in decreased viability and recovery. For this, transferring the cryovials to liquid nitrogen within 2 days is recommended.
6. Cells that die during the freeze/thaw procedure release DNA strands that can cause cell clumping and aggregation. Therefore the use of a DNase-containing collection medium is recommended while thawing PBMC.
7. Because of cell viability it is of utmost importance to thaw and process cells rapidly. Thawing the cryovials should be done in approximately 1 min and never takes longer than 2 min.
8. It is advisable to dry and wipe the outside of the vial with 70% ethanol to minimize the chance of contamination.
9. Gradual dilution of DMSO avoids the osmotic shock, and the warm temperature assures that the cells can compensate the decreasing osmotic pressure.
10. We demonstrated previously that the freeze/thaw cycle affects protein expression efficiency dramatically after mRNA electroporation (8). Indeed, an overnight resting period of thawed PBMC before electroporation is required to allow efficient protein expression as was shown by the absence of transgene expression when mRNA electroporation was done directly after thawing in contrast to inclusion of an overnight resting phase before electroporation (Fig. 1).
11. By using these settings, it has been demonstrated previously that mRNA could be efficiently transfected into resting PBMC (8, 23), while transfection efficiency as well as cell viability decreased upon the use of different settings. For this we prefer electroporation devices that allow the operator to freely install any settings wanted. Follow the suggested guidelines that come with your electroporation device.
12. We have experienced that it is mandatory to use phenol red pH indicator-free OptiMEM for electroporation purposes in order to yield optimal cell viability.
13. Dependent on the trial and/or application mRNA encoding various antigens can be used. mRNA encoding the antigen of

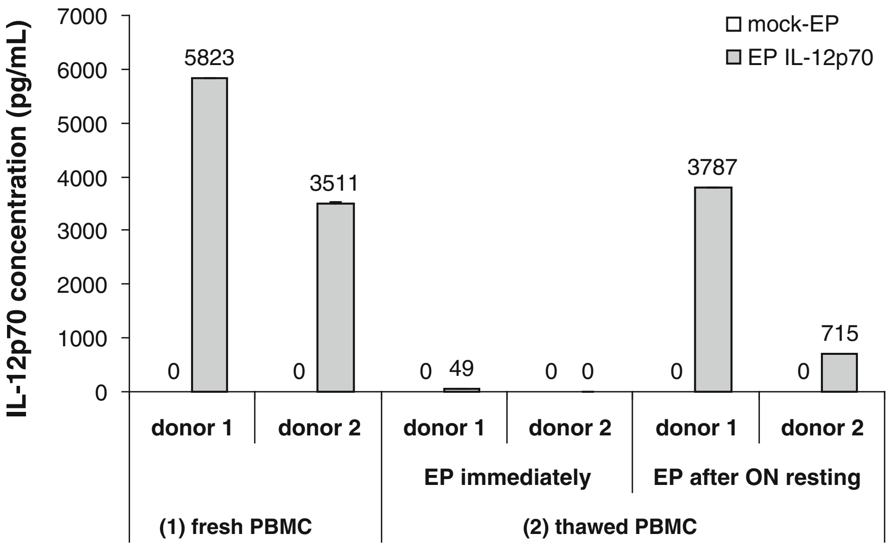

Fig. 1. IL-12p70 secretion in fresh and cryopreserved PBMC. Unstimulated fresh PBMC were (1) electroporated with IL-12p70-encoding mRNA (fresh PBMC), (2) frozen and electroporated after thawing (thawed PBMC), either immediately (EP immediately) or after overnight resting (EP after ON resting). Secretion of IL-12p70 was measured 24 h after electroporation. Each bar represents the mean concentration of duplicates ± SD.

interest can be (a) directly isolated from the target cell (25), organ or tumor (26), (b) in vitro transcribed starting from cDNA using specialized kits (e.g., mMessage mMachine kit, Ambion or mScript mRNA Production System, Epicentre Biotechnologies), or (c) directly purchased from a specialized company (e.g., CureVac GmbH, Tubingen, Germany). The described procedure is similar for every kind of mRNA used.

14. As a control mock electroporation (w/o addition of mRNA) or electroporation with irrelevant mRNA can be used.
15. It is recommended to electroporate a minimum of 5×10^6 PBMC per electroporation and a maximum of 50×10^6 PBMC.
16. Working with mRNA requires special attention to detail. mRNA is best stored frozen, preferably at −80°C and should be kept on ice whenever possible. The primary concern is nucleases. For this reason all buffers, pipets, pipet tips, tubes, and anything else that will come into contact with the mRNA should be autoclaved and subsequently handled as a sterile solution. Because fingers are an excellent source of nucleases, you should be careful not to touch anything with bare fingers. RNA is usually stored in distilled water which has been treated with diethyl-pryocarbonate (DEP) to inactivate RNases. Many RNases survive autoclaving, so DEP should be used to treat solutions whenever possible.
17. The most optimal concentration of mRNA to work with during the electroporation procedure is 1 μg mRNA/μL. Use 10 μg

mRNA per electroporation for ≤20 × 10^6 PBMC per electroporation. Use 20 μg mRNA per electroporation for >20 × 10^6 PBMC per electroporation.

18. Except for the development of the Elispot plates, all work needs to be performed under a biological safety cabinet following standard biosafety level 2 precautions and all work needs to be performed using sterile techniques.
19. Dependent on the trial and/or application a variety of cytokines can be tested. Anti-cytokine Elispot kits are commercially available from a multitude of suppliers or can be developed and optimized by the operator.
20. Our experience is that AT LEAST 2.5 × 10^5 PBMC/well are necessary for optimal detection of cytokine-producing cells. Dependent on the antigen and cytokine to be tested these numbers need to be optimized and adjusted.
21. Take care that the position of the plates in the incubator is leveled. If not, cells will roll towards one side of the well resulting in so-called moon-like shapes and thereby comprising the result of the assay.
22. To dry the membrane of a PVDF plate is an essential step. We have found it helpful to dry plates upside-down in the air flow of a biosafety cabinet.
23. Others have intensively developed useful guidelines for performing elispot assays and analysis (27–29).

Acknowledgments

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Chapter 19

mRNA PCR-Based Epitope Chase Method

Jean-Daniel Doucet, Dominique Gauchat, and Réjean Lapointe

Abstract

The identification of specific viral and tumor antigen epitopes recognized by CD4$^+$ or CD8$^+$ T lymphocytes remains a challenge. Unfortunately, epitope mapping methods are generally costly and time-consuming. This chapter details a polymerase chain reaction (PCR)-based mRNA epitope identification method called mPEC, which is designed to rapidly and precisely identify relevant T cell epitopes recognized by previously isolated CD8$^+$ or CD4$^+$ T lymphocytes.

This method is based on the use of mRNA fragments synthesized from PCR-amplified cDNA with a variety of 3′end iterative deletions. mRNA fragments are electroporated into autologous antigen-presenting cells to map the epitope in a given protein antigen. Considering mRNA's sensitivity to degradation, we also insert a control define epitope at the mRNA's 3′end to control for electroporated mRNA's integrity and capacity to be translated.

Key words: Major histocompatibility complexes class I and II, T cell epitope, Epitope mapping, mRNA transfection

1. Introduction

There is a clear need in viral (1) and tumor (2) immunology to find a wide array of antigens that can be targeted by the cellular arm of the immune system, involving CD8$^+$ cytotoxic and CD4$^+$ helper T cells recognizing epitopes presented by major histocompatibility complex (MHC) classes I and II.

Three prerequisites are essential for validating an epitope as a prophylactic or therapeutic cellular immune response target (3).

1. First, an epitope must be processed by antigen-presenting cells (APCs). Some epitopes are degraded by cellular proteases and are not presented in vivo.

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2. An epitope must also have good affinity for its MHC molecule, which keeps it presented on the surface of APCs and triggers circulating T cell response.
3. Finally, an epitope must stimulate specific T cell receptors (TCRs). Some epitopes are found at the surface of APCs, but are not well enough presented by APCs or are not targeted by any specific T cells.

Several approaches have been developed to map isolated T cell epitopes (1, 4). Peptide loading technologies (i.e., peptide libraries and recent ultraviolet light-dependent MHC-peptide exchange technologies (5)), mass spectrometry analysis of peptides bound to MHC molecules (6), and screening of cDNA libraries (7) have identified many useful T cell epitopes in different diseases. However, these techniques are often time-consuming and may sometimes not reflect genuine epitopes recognized by specific T lymphocytes (4).

As an alternative, mRNA fragments have been used to characterize the specificity of previously isolated T cells (8). This technique takes advantage of plasmid DNA templates cleaved at available restriction sites, or at new sites inserted by site-specific mutagenesis, from which mRNA fragments of various lengths are generated. However, it is limited by the position of restriction sites, and expression controls are absent.

mRNA PCR-based epitope chasing (mPEC) is a significant improvement in mRNA fragment-based human T cell epitope mapping. Like other techniques, it uses previously isolated and expanded T cells from human leukocyte antigen (HLA)-typed peripheral blood mononuclear cells (PBMCs) (9) to map genuine epitopes (see Fig. 1). Such T cells are expanded by coculture with CD40-activated B lymphocytes (CD40-B) cells as APCs expressing an antigenic protein.

mPEC further uses PCR-amplified cDNA fragments of various lengths as templates for mRNA synthesis, circumventing the need for restriction site insertion and allowing for great flexibility. It also allows addition of control epitopes, such as the M1$^{58-66}$ epitope (GILGFVFTL) that serve as internal positive control of mRNA electroporation efficiency.

Resulting mRNA fragments are electroporated into autologous B lymphocytes immortalized by Epstein-Barr virus (EBV-B), thereby making it possible to match haplotypes exactly, in contrast to most current epitope identification techniques. In the same experiment, it is also possible to identify both CD4 and CD8 epitopes. Electroporated autologous EBV-Bs are then used as APCs in a coculture with expanded T cells to deduce the epitope localization; mRNA fragments in which the epitope sequence is deleted are not recognized by specific T cells.

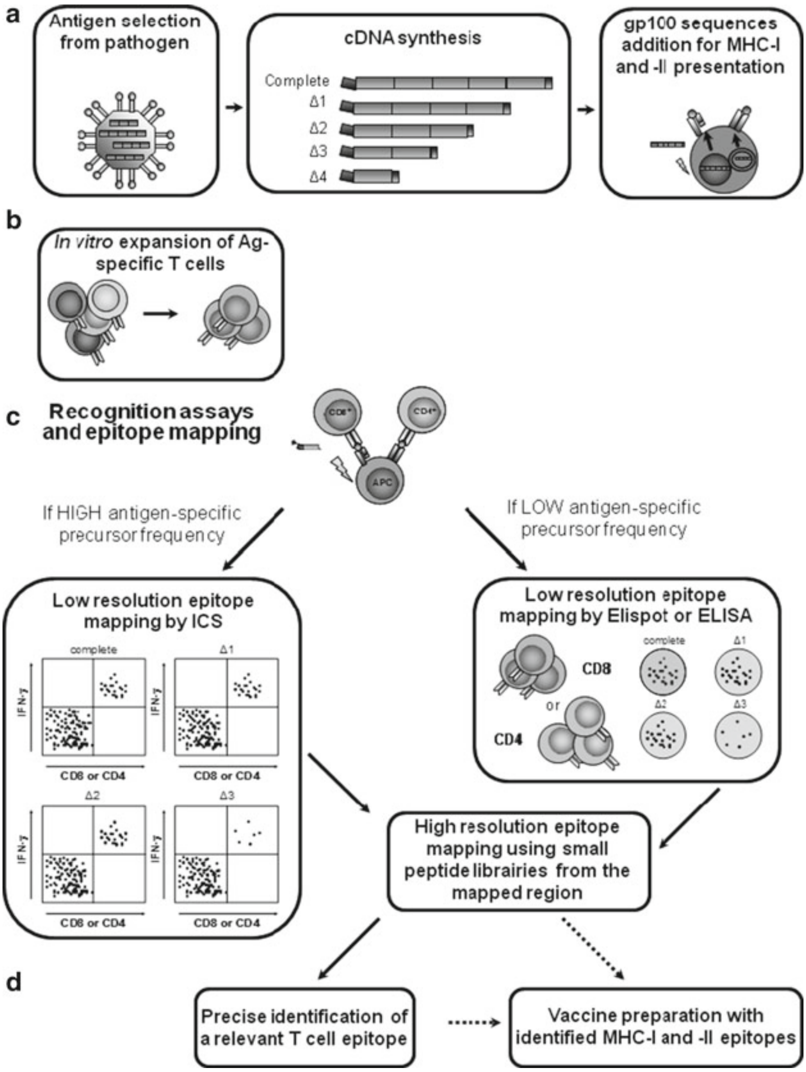
a
Antigen selection from pathogen
cDNA synthesis
Complete
Δ1
Δ2
Δ3
Δ4
gp100 sequences addition for MHC-I and -II presentation
b
In vitro expansion of Ag-specific T cells
c
Recognition assays and epitope mapping
CD8+
CD4+
APC
If HIGH antigen-specific precursor frequency
If LOW antigen-specific precursor frequency
Low resolution epitope mapping by ICS
complete
Δ1
Δ2
Δ3
IFN-γ
CD8 or CD4
Low resolution epitope mapping by Elispot or ELISA
CD8
or
CD4
complete
Δ1
Δ2
Δ3
High resolution epitope mapping using small peptide libraries from the mapped region
d
Precise identification of a relevant T cell epitope
Vaccine preparation with identified MHC-I and -II epitopes

Fig. 1. Schematic of the mPEC epitope mapping technique. (**a**) *Antigen selection and cloning*. Antigens and successive deletants are cloned in an expression plasmid in fusion with MHC class II targeting sequences to optimize presentation by MHC class II if needed (14) and with a control MHC class I epitope from a known epitope. (**b**) *Electroporation of APCs.* Briefly, the optimized antigens and successive deletants are electroporated in autologous APC (either CD40-activated or EBV-immortalized B lymphocytes). Autologous blood T lymphocytes are cocultured with electroporated APC. (**c**) *Low resolution epitope mapping.* Epitope recognition by blood T lymphocytes can be achieved by two methods of varying sensitivity: Enzyme-linked immunosorbant spot assay (ELISPOT) and intracellular cytokine staining (ICS) to (1) determine if CD4$^+$, CD8$^+$, or both T cell types recognize epitopes and (2) localize the epitope by a modified mPEC method (US provisional patent application and (4)). (**d**) *Final epitope identification.* Epitopes are further identified by synthesis of peptides covering the shortest fragment recognized by relevant T cells by mPEC with overlapping peptide libraries.

Once a small epitope “hot spot” is narrowed down by this iterative process, smaller peptide libraries may be used to determine the precise epitope sequence. This strategy has made it possible to rapidly and specifically identify 3 epitopes, 2 MHC class I and 1 MHC-II, from influenza antigens recognized by previously isolated and expanded antigen-specific $CD8^+$ or $CD4^+$ T lymphocyte (4).

2. Materials

2.1. Cell Culture Reagents

1. RPMI 1640 complete medium: Add 50 ml (10%) of fetal bovine serum (FBS), 5 ml of 200 mMl-glutamine (2 mM final), 5 ml of a 10,000 IU penicillin (100 U/ml final) and 10,000 μg/ml streptomycin (100 μg/ml final) solution, and 100 μl of 50 mg/ml gentamicin (10 μg/ml final) to a 500 ml bottle of RPMI 1640 1× with l-glutamine.
2. Iscove's modified Dulbecco's complete medium: Add 37.5 ml (7.5%) of uncomplemented (heat-inactivated, 55°C for 30 min) human serum AB (Cat. Number 100-512; GemCell), 5 ml of 200 mMl-glutamine (2 mM final), 5 ml of a 10,000 IU penicillin (100 U/ml final) and 10,000 μg/ml streptomycin solution (100 g/ml final), and 100 μl of gentamicin (10 μg/ml final) to a 500 ml bottle of Iscove's modified Dulbecco's medium 1×.
3. Human IL-2 working solution: Resuspend 16.9×10^6 IU (1 mg) in 1 ml of sterile 100 mM acetic acid, then add 26 ml of Iscove's modified Dulbecco's medium (600,000 IU/ml final).
4. 96-, 48-, 24-, and 12-well plates.

2.2. Plasmid and Electroporation Components

1. pcDNA3.1(+)-(gene-of-interest).
2. Neon® transfection system microporator, (Invitrogen, Cat. Number MPK5000).
3. Neon® 100 μl transfection system kit, including resuspension buffer, electrolyte buffer, disposable gold-tips, electroporation pipette, and microporation tubes (Invitrogen, Cat. Number MPK10096) all compatible with the Neon® transfection system microporator.

2.3. PCR Amplification

1. Platinum® *Pfx* DNA polymerase kit, including a 50 mM $MgSO_4$ solution and 10× pfx Amp. buffer (Invitrogen, Cat. Number 11708-013).
2. Primer resuspension: Resuspend T7 forward (5′-TTAATACGACTCACTATAGGG-3′), BgH reverse (5′-TAGAAGGCACAGTCGAGG-3′), and all other relevant primers for specific gene of interest at 0.1 mM in sterile and RNase-free water (stock solution). Add 20 μl of 0.1 mM primer solution to 80 μl of sterile and RNase-free water in a new 0.6 ml Eppendorf tube (20 μM working solution).

3. Polymerase chain reaction (PCR) mix: Thaw reagents on ice. Mix in a 0.6 ml PCR tube 5 μl of 10× Hifi buffer, 1 μl of 10 mM dNTP, 40 μl of RNase-free water, 1 μl of 50 mM $MgSO_4$, and 1 μl of each 20 μM primer in a designed PCR hood. Quickly add 0.5 μl of 2.5 U/ml *Pfx* Taq polymerase. Out of the hood, add 0.5 μl of 0.1 μg/μl of template DNA.
4. 1.5% Agarose gel: Add 1.5 g of (RNase-free) agarose to 100 ml of (RNase-free) water. Heat the mix until it boils. Pour the mix into a migration mold.
5. 1 kb DNA ladder.
6. Illustra GFX™ PCR DNA and gel band purification kit (GE Healthcare, Cat. Number 28-9034-70).

2.4. In Vitro Transcription mRNA Components

1. mMessage mMachine T7 capped RNA transcription kit (Ambion, Cat. Number AM1344).
2. MEGAclear™ purification of transcription reactions kit (Ambion, Cat. Number AM1908).
3. Poly(A)tailing kit (Ambion, Cat. Number AM1350) (see Notes 1).

2.5. DNA and mRNA Electrophoresis

1. Agarose from your standard supplier.
2. Standard electrophoresis equipment.
3. 1 kb DNA ladder (Invitrogen, Cat. Number 10787-018) (for DNA electrophoresis).

2.6. T Cell Assay Components

1. 96-Well plates round bottom.
2. 9-mer (MHC-I) or 15-mer (MHC-II) peptide sequences. Final M1-CD8 T cell clone peptide: GILGFVFTL. Final NP-CD8 T cell clone peptide, LSAFDERRNKYLEEHPSAG, Final M1-CD4 T cell clone peptide, NGNGDPNNMDKAVKL (see Note 2).
3. Peptide resuspension: Resuspend peptides at 10 mg/ml (stock solution) in DMSO.

   Add 10 μl of 10 mg/ml of 9-mer peptide (MHC-I) to 90 μl of new DMSO (1 mg/ml working solution) or 10 μl of 10 mg/ml 15-mer peptide (MHC-II) to 10 μl of DMSO (5 mg/ml working solution).
4. Human IFN-gamma DuoSet ELISA kit (RnD, Cat. Number DY285).
5. Human IFN-gamma ELISPOT kits (RnD, Cat. Number EL285).
6. Blocking antibodies specific for MHC-I (clone W6/32) or HLA-DR (clone HB55) (if needed).

2.7. HLA Typing Service

1. Local HLA typing service or,
2. Commercial kit, i.e., Invitrogen Cat. Number 5300925,

3. Methods

Carry out all procedures at room temperature unless otherwise specified. All reagents used in the presence of mRNA must be dedicated and kept in RNase-free conditions. Carry out all procedures involving T cells, APCs, and PBMCs using standard aseptic techniques.

3.1. PBMCs and Cell Lines

1. Obtain and separate PBMCs from donor's heparinized blood. Healthy individuals were informed and consent using a routine technique. Cryopreserve in liquid nitrogen many vials of PBMCs in 90% FBS/10% DMSO (Sigma). Keep some of them for the next step or for T cell expansion if needed (see further).
2. Immortalize PBMCs B lymphocytes with Epstein-Barr virus (EBV-B cells). This procedure should be performed in a P2 environment (10).
    - (a) Cultivate B95-8 cells (CRL 1612, ATCC, Rockville, MD) in complete RPMI medium containing 15% FBS at 10^6 cells per ml initial concentration.
    - (b) Split 1/2 when medium turn yellow, then after the second split, transfer 5 ml in 30 ml of fresh medium in a T75 flask, repeat for the third split, spin down at 300 *g* for 7 min, filtrate the supernatant through a 0.45 μm filter, aliquot and freeze at −180°C. These supernatants contains live infectious virus.
    - (c) Place 10^7 donors PBMC in 5 ml of RPMI complete medium in a T25 flask, add 5 ml of the above produced B95-8 supernatant + cyclosporine A (Sandoz) at 0.5 μg/ml. Incubate the flask in a upright position for 7–10 days until clumps are visible (it could take up to 3 weeks).
    - (d) Split initially 1/2, and later according to growth rate (when medium turn yellow). After the second split, wash cells twice in PBS to eliminate residual free viruses and to be able to leave the P2 (see Notes 3).

3.2. Plasmids

1. Clone the genetic sequence of the antigenic protein of interest into a plasmid downstream of a T7 promoter and transform it in *E. coli* competent cells (see Notes 4 and 5).

3.3. Electroporation of APCs

1. Culture EBV-B for 3–4 days using above-mentioned procedures.

2. Sediment EBV-B for 15 min at 100 × *g*, resuspend in electroporation resuspension buffer with 3–4 μg/10^{-6} cells of DNA or mRNA.
3. Electroporate using an efficient B cell electroporation protocol and resuspend the cells in RPMI 1640 (EBV-B) or Iscove's modified Dulbecco's complete (CD40-B) medium without antibiotics (see Note 6).

3.4. Generation of Antigen-Specific T Cell Clones

1. Stimulate PBMCs ($0.6–1 \times 10^6$) with $0.3–0.5 \times 10^6$ autologous CD40-activated B cells (see Note 7). On days 6–8, restimulate bulk T cells following the same procedure, and add 150–300 IU/ml of IL-2 (Feldan) on the following day and every 2–3 days (9).
2. Test T cells on day 20–21 for their specificity on the basis of IFN-γ secretion (see recognition assays below).
3. Clone antigen-specific bulk T lymphocytes by limiting dilution (10).
   (a) Prepare the following mix: 100 ml Iscove's modified Dulbecco's complete medium, 1×10^6 ml^{-1} irradiated PBMCs (5,000 rad) prepared from three different normal donors, 30 ng/ml anti-CD3 (OKT3), 300 U/ml hrIL-2, and $2–10 \times 10^3$ bulk T cells.
   (b) Add 100 μl of the previous cell preparation, to plate 1–10 cells/well in 96-well round bottom
   (c) Culture for 1 week at 37°C, 5% CO_2.
   (d) Add 100 μl per well of Iscove's modified Dulbecco's complete medium containing 30 ng/ml anti-CD3 (OKT3), and 300 U/ml hrIL-2.
   (e) Culture for 1–2 week at 37°C, 5% CO_2 until a sufficient numbers are available.
   (f) In wells containing big pellets of growing cells (medium should turn orange to yellow), evaluate 50% of the cells in a recognition assay for specificity (see recognition assays below).
   (g) Expand T lymphocytes displaying a strong recognition of the antigen (over a negative control using target cells expressing empty vector or an irrelevant antigen) in number with the Rapid expansion protocol.
4. Rapid expansion protocol
   (a) Relevant T cell clones (see Subheading 3.6) are expanded in T-25 tissue culture flasks (kept in vertical position) at $1–5 \times 10^5$ cells/flask with 2.5×10^7 irradiated PBMC feeders prepared from three different donors and 30 ng/ml anti-CD3 in 25 ml of Iscove's modified Dulbecco's complete medium.

   (b) On day 2, add 300 U/ml rIL-2, and on day 5, replace 20 ml of medium with fresh Iscove's modified Dulbecco's complete medium containing 300 U/ml rIL-2.
   (c) On day 8, aspirate 20 ml of medium and count the cells in the remaining 5 ml of medium. For the remaining culture, keep the cell concentration at $1–2 \times 10^6$ cells/ml in Iscove's modified Dulbecco's complete medium with 300 U/ml rIL-2.
   (d) After 14–28 days of culture, evaluate their specificity as before by coculture with the gene coding for the protein of interest, in our case influenza matrix protein 1 (M1) or nucleoprotein (NP)-electroporated EBV-B cells on the basis of their IFN-γ secretion (see Subheading 3.6).
   (e) Stain T cell clones with anti-CD3, CD4, and CD8 antibodies suitable for flow cytometry analysis.
   (f) Determine the phenotype (CD3, CD4, or CD8) of the T cell clones specifically responding to the antigen using flow cytometry.
   (g) Cryopreserve in liquid nitrogen antigen-specific T cell clones in 90% FBS/10% DMSO (see Note 8).

3.5. In Vitro Transcription of mRNA

1. Amplify the antigenic protein-coding cDNA fragments by standard PCR from the relevant DNA plasmid template with high-fidelity platinum® *Pfx* DNA polymerase. Use a 5′end primer including the T7 promoter, such as the T7 forward primer (5′-TTAATACGACTCACTATAGGG-3′) and 3′end primers creating successive 3′end deletions starting from a BGH reverse primer (5′-TAGAAGGCACAGTCGAGG-3′) for example (see Notes 9 and 10).
2. Synthesize mRNA in vitro using the mMessage mMachine® kit (Ambion). Poly-adenylate the resulting RNA with the poly(A) tailing kit (Ambion). Finally, purify mRNA with the MEGAclear™ kit (Ambion). All steps are performed according to the manufacturer's instructions.
3. Migrate sample mRNAs in a 1.5% agarose gel at 100 V for around 15 min to approximately confirm integrity (see Note 11).

3.6. APC Pulsing and Recognition Assays

1. Thaw, wash, and culture EBV-B 3–4 days before the assay.
2. Thaw and culture T cell clones for a 4 h recovery time at 37°C, 5% CO_2 in an incubator in Iscove's complete medium supplemented with 120 IU/ml IL-2.
3. Prepared target cells with peptide pulsing or antigen electroporation
   (a) Peptide-pulsed EBV-B for 3 h at 37°C 5% CO_2 (e.g., Final M1-CD8 T cell clone peptide: GILGFVFTL. Final NP-CD8

T cell clone peptide, LSAFDERRNKYLEEHPSAG, Final M1-CD4 T cell clone peptide, NGNGDPNNMDKAVKL). Add synthetic peptides at a final concentration of 1–10 μg/ml for MHC class I 10-mer peptides (50 μg/ml for peptides longer than 14 amino acid) to EBV-B cells plated at 0.5–1 × 10^6 cells/ml.

   (b) Antigen-electroporated EBV-B: after overnight rest were electroporated according to protocol explained in steps in Subheading 3.3.

4. Wash peptide-pulsed or electroporated EBV-B in PBS 1× and resuspend them in Iscove's modified Dulbecco's complete medium.
5. Coculture pulsed or electroporated EBV-B cells with T cell clones targets at a 1:1 ratio (usually 30,000–50,000 cells each) in a round bottom 96-well plate for 20 h at 37°C, 5% CO_2.
6. To evaluate the reactivity to MHC-restricted epitopes, measure IFN-γ secretion by ELISA using the manufacturer's kit protocol (9) (see Note 12).

3.7. MHC Typing of Donor PBMCs

1. Genotypes and serotypes PBMCs using an HLA typing service.
2. From the MHC haplotype obtained, use MHC-matched EBV-B cells from other normal donors in peptide-pulsed recognition assays (see Subheading 3.6).
3. Deduce epitope MHC restriction element from MHC-matched EBV-B cells allowing T cell clone peptide recognition (see Note 13).

3.8. MHC Class I and II Epitope Validation Using the mPEC Method

1. Validate the epitope by loading identified peptides as described earlier directly on PBMCs from other MHC-matched donors.
2. Culture 100,000–400,000 peptide-loaded PBMCs per well of a 96-well plate pre-coated with anti-IFN-γ antibodies from routine sensitive ELISPOT kits at 37°C, 5% CO_2 for 6–12 h.
3. Evaluate specific IFN-γ secretion following the manufacturer's instruction (see Note 14).

4. Notes

1. You can also use Ambion's AM 1345 kit which includes both mMessage mMachine T7 capped RNA transcription kit and Poly(A)tailing kit.
2. There are many peptide synthesis companies providing excellent purity for T cell assays available at minimal cost. For

complete peptide sequences we used for T cell clone epitope mapping, we refer to our previous article supplementary methods (4).

3. CD40-activated B (CD40-B) cells generated could be used as an alternative for EBV-B cells.

   Recombinant soluble CD40L (1,000 ng/ml) from the former Immunex Corporation was used in our experiments, but rhsCD40L from Enzo or irradiated 3 T3-CD40L cell lines could also be used. Add rhsCD40L and recombinant human interleukin-4 (IL-4, 250 U/ml) to PBMCs on the first day in Iscove's modified Dulbecco's complete medium. Alternatively, enriched B lymphocytes can be used. On day 3, add again fresh Iscove's modified Dulbecco's complete medium containing 250 U/ml IL-4 and 1,000 ng/ml CD40L. After the first round of proliferation (days 5–8), cells can be either frozen for future use or can be restimulated every 2–3 days when the culture reached a density of $1.5–2 \times 10^6$ cells/ml. After 8 days of culture, more than 70% of the cells should express both CD19 and HLA-DR surface markers. For further details on CD40-B cells profile, these articles may be consulted (11, 12).

4. DNA sequences optimized for improved expression in mammalians give much higher protein expression in cells, both with plasmid and in vitro transcribed mRNA. For example, we used NP and M1 matrix proteins from influenza virus A/Puerto Rico/8/1934/H1N1 strain (uniprot # P03466 (NP) and P03485 (M1)) optimized for improved protein expression by GeneOptimizer© from Geneart. Many companies offer these services at low cost.

   Subcloning into pcDNA3.1(+) plasmid (Invitrogen) was also performed by Geneart. pcDNA3.1(+) is an relevant plasmid because it includes, before the multiple cloning site, the T7 promoter (TTAATACGACTCACTATAGGG) used for cDNA PCR amplification and subsequent T7 polymerase-mediated mRNA synthesis. Other expression plasmids could be used.

   If required, a T7 promoter could be added during the PCR amplification step at the 5′end of the forward primer. Plasmids were then transformed into *E. coli* One Shot© TOP 10 competent cells (Invitrogen) and prepared by plasmid Megaprep kit (Qiagen).

5. Classically, MHC class II epitope originate from external antigens that are taken up by endocytosis. Influenza M1 and NP antigens used in our study (4) are presented by MHC-II even if they were endogenously expressed inside CD40-B and EBV-B cells (13). This is allowed us to use the mPEC technique to directly map M1-specific $CD4^+$ T cell clone specificity. However, other proteins may not be adequately presented by

MHC-II when endogenously expressed. In such cases, MHC-II mobilization sequences must be added to the antigen of interest to mobilize the protein in endosomal compartments, as we (13, 14) and others (15, 16) have described. In other words, MHC class II mobilization sequences must be added to the original DNA template. For example, the putative N-terminal signal sequence (first 23 residues) and the last 84 residues from gp100 could, respectively, be added to the N-terminal and C-terminal of the antigenic protein (14). Alternatively, mobilization sequences from the invariant chain could also be used (17). To simplify the process, we recommend adding these sequences to the gene of interest in the initial plasmid DNA template.

6. For both EBV-B and CD40-B lymphocytes, an MP-100 microporator (Digital-bio/Invitrogen) was used according to the same protocol of 1 pulse at 1,700 V for 20 ms. The rest of the electroporation procedure was followed according to the manufacturer's instructions. Transfection efficacy ranges from around 10–40%. EBV-B cells have slightly higher transfection efficacy and antigen expression than CD40-B cells (see Fig. 2). Other standard and efficient transfection methods can also be used as alternatives.

   Do not use antibiotics in the culture medium to resuspend B cells in the hours following their electroporation. Electroporation makes cells more vulnerable to antibiotics and cells are much more likely to die.

7. If antigen-specific T cell frequency is too low, expand them using a T cell protocol, as briefly described in this chapter. Other T cell expansion protocols may also be used (Miltenyi biotec's MACS expansion of antigen-specific cytokine-secreting T cells protocol or using IL-2, -7, and -15 cytokines (18)). Usually, around days 20–21, maximal T cell expansion is obtained before activation-induced cell death. The 14–28 days window of expansion for T cell clones is used for this same reason. Thus, for T cell expansion, we strongly recommend using CD40-B cells as APCs and not EBV-B, since the latter may stimulate EBV-specific T cells. Such a procedure merits its own chapter and therefore goes beyond the scope of mPEC epitope mapping techniques.

   Also, the frequency of antigen-specific T lymphocytes in the bulk culture can be as low as 1%, and sometimes, can be very high. As a routine procedure, it is then a good practice to clone bulk antigen-specific T lymphocyte cultures at 2 cells/well in 10 plates, 5 cells/well in 10 plates, and 10 cells/well in 2 plates. These numbers can vary according to the empirical capacity to obtain enough individual growing clones. It is also recommended to test wells from plates with less than 10 growing

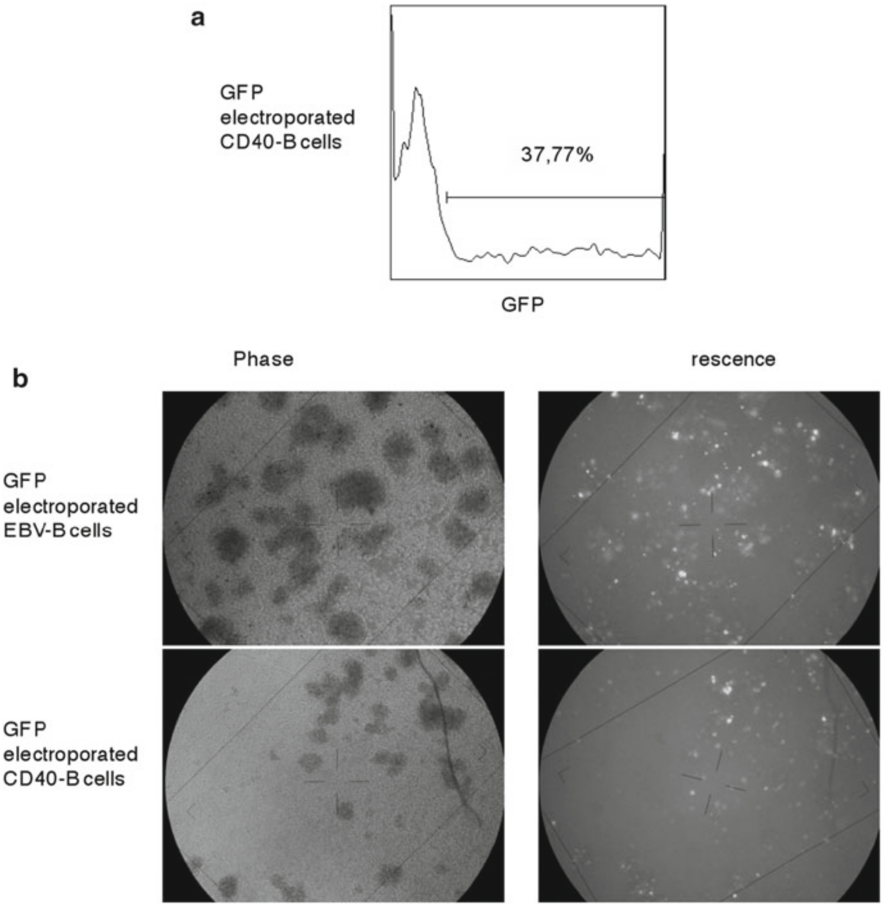

Fig. 2. Expression of GFP in pcDNA3.1-GFP plasmid electroporated CD40-B cells and EBV-B cells. (**a**) Expression of GFP expression in pcDNA3.1-GFP electroporated in healthy and proliferating CD40-B cells as determined by flow cytometry. (**b**) Expression of GFP expression in pcDNA3.1-GFP electroporated in CD40-B cells compared to CD40-B as observed by fluorescence microscopy.

wells/plate, since too many growing wells per plate could indicate that more than one clone are growing per well. The goal is to screen from 50 to 200 individual wells where proliferation is evident (growing pellet and medium turning orange to yellow) to find antigen-specific clones.

8. Antigenic recognition should be evaluated at different time during the expansion procedure from day 10, and cultures should be interrupted when T cell clones stop to proliferate and decline. T cell lines worth cloning should generally have secretions of more than 400 pg/ml of IFN-γ in the above-mentioned assay conditions and secrete twice as much IFN-γ as the negative control. A good T cell clone should have low background (hundreds of pg/ml) and secrete more than 1,000 IFN-γ/ml, MIP-1β, or other pro-inflammatory cytokines can also be used as readouts (13).

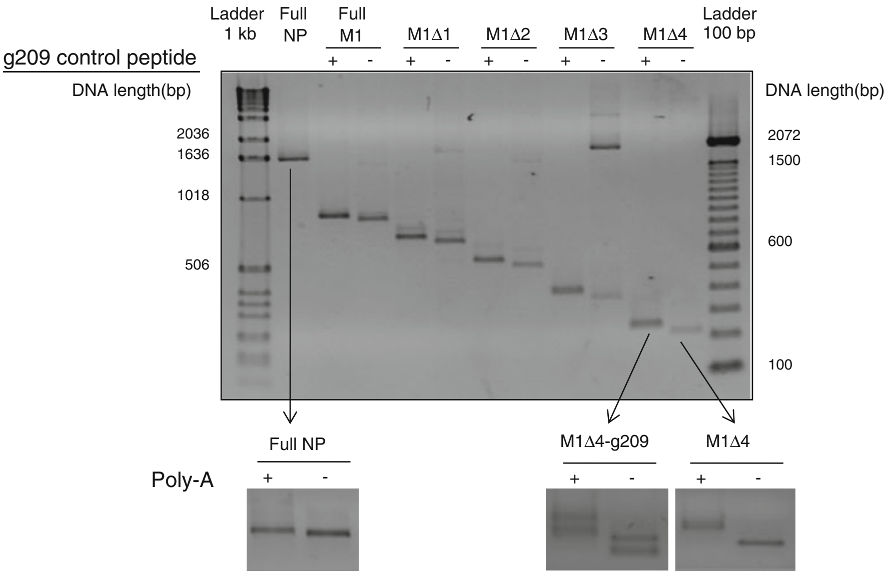

Fig. 3. M1 PCR-amplified cDNA fragments obtained from plasmid template. M1 PCR-amplified cDNA fragments with or without 3′end gp100$^{209–218/2M}$ control peptide (g209) were migrated on 1.5% agarose gel for 1 h. Migration of some M1 RNA fragments synthesized from M1 cDNA fragment templates, with or without subsequent poly-adenylation, was performed for 15 min on 1.5% agarose gel in non-denaturing, non-RNase-free conditions.

T cell clones can then be re-expanded according to the same protocol, or frozen. Routinely, with vigorous T cell clones and in optimal conditions, it is possible to obtain from 10 to 100×10^6 T cells. However, each clone responds differently and sometimes, don't respond at all. Experience will help to optimize the yield of antigen-specific T cell clones from this complex procedure.

9. Standard pcDNA3.1(+)-M1, -NP, or -mock were used with high-fidelity platinum® *Pfx* DNA polymerase. pcDNA3.1(+) has the advantage of containing a T7 promoter just in front of the multiple cloning site that can be used for mRNA synthesis.

   Our default cycling conditions are 15 min at 95°C, followed by 35 cycles of 45 s at 94°C, 45 s at 55°C, and 90 s at 72°C. The primer sets need to be chosen carefully. Unspecific fragments are sometimes amplified by specific sets of primers. We recommend evaluating 2 or 3 different primer sets for every cDNA deletion to make sure at least one set amplifies only the specific fragment. Alternatively, if multiple fragments are amplified, cDNA templates can be isolated on preparative agarose gel and re-amplified by PCR to ensure purity, as done previously with homemade or commercial kits (i.e., GFX™ PCR DNA and gel band purification kit) (see Fig. 3).

10. While amplifying cDNA templates, insert a control epitopes such as the M1$^{58–66}$ epitope (GILGFVFTL) at the 5′end of reverse DNA primers, before stop codons. Such epitopes serve as internal positive control of mRNA electroporation efficiency with pregenerated control T cell clones. RNases are ubiquitous and can often cause mRNA degradation. The results with negative response on control epitopes must be rejected as false negative, even though mRNAs could be detected on agarose gel.

    We chose nucleotide sequences of the HLA-A2 restricted influenza M1$^{58–66}$ epitope and the g209 melanoma epitope, for which we possess antigen-specific T cells. HLA-A2$^+$ individuals have high frequencies of M1$^{58–66}$-specific T cells. Other epitopes may be used depending on available T cell clones and on HLA haplotype. If you are using fresh PBMCs, T cells specific to these control epitopes will raise the background of T cell frequency. In that case, subtract the control antigen-specific signal from the signal of your antigen of interest-specific T cell frequency.

    Aliquot the quantity of at least two electroporations of mRNA per 0.6 ml Eppendorf tubes at 1 mg/ml to make sure you have enough for more than 1 per tube and minimize mRNA freeze and thaw cycles. 6 μg (6 μl) per tube should be enough since roughly 3 μg are required for a standard electroporation. This will ensure that you have just the right amount of mRNA per tube and avoid potential RNase contamination of mRNA.

11. Non-denaturing/non-RNAse-free gel may also be used as a general overview of RNAs quality, as the control epitope added previously will confirm final protein production by the mRNA.

12. To approximately screen HLA restriction of the T cell clone, pre-incubate EBV-B or CD40-B cells for 20 min at 37°C, 5% CO_2 with 40–80 μg/ml of blocking antibodies specific for either MHC-I (clone W6/32) or HLA-DR (clone HB55) to infer the presence of a CD8$^+$ or a CD4$^+$ T cell clone.

13. We used our local Laboratoire d'histocompatibilité, INRS-Institut Armand-Fappier (Laval, Quebec, Canada). HLA genotype of PBMCs from our normal donor was HLA-A*02, 33; B*35, 51; Cw*04, 16; DRB1*04, 11; DQB1*03, 03. HLA-typing kits are also commercially available (i.e., Invitrogen Cat. Number 5300925). In our case, we identify HLA-A*02 as a restriction element for a M1-specific CD8$^+$ T cell clone using the T2 cell line, sharing only HLA-A*02 haplotype with our normal donor.

14. Briefly, use directly PBMCs instead of expanded T cell clones. Since the epitope was already validated once, T cell directly from PBMCs can directly be used to determine specific T cell frequencies in individual using routine ELISPOT assays easily available in kit from many manufacturers. You should expect to

see more spots when a valid peptide is added, when compared to un-pulsed PBMC or pulsed with irrelevant peptides. As a positive control, CEF or addition of PHA (Sigma, L2769) could be used. HLA-matched donors should have T cell frequencies around 0.05–0.5%, depending on the antigen studied. If antigen-specific T cells are too low, proceed to T cell expansion as explained earlier in this chapter. The point is to confirm that the epitope is common in a human population.

5. Discussion

We have developed a rapid, accurate and adaptable method called mPEC for mapping and defining the specificity of either MHC class I or II epitopes, even though MHC class II epitopes have traditionally lacked effective identification techniques (4).

PCR-amplified cDNA templates for mRNA synthesis allow the precise identification of T cell epitopes. Interestingly, as our technique uses autologous activated B lymphocytes as APCs, it does not require previous knowledge of the MHC restriction element. However, if required, specific epitope MHC restriction elements can be identified by established methods using blocking antibodies and a panel of EBV-B cells from donors of various HLA allelic variants (4, 19). Expression in APCs also allows the identification of T cell epitopes that will most likely be processed in vivo in contrast to other epitope identification techniques. Overall, the mPEC technique is an easy and precise tool for discovering new in vivo-relevant T cell epitopes from previously isolated T cells.

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